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Establishment of an *in vitro* model to identify the molecular mechanism of immortalisation by MLL-ENL.

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Abstract

The t[(11;19)(p22;q23)] translocation, which gives rise to the MLL-ENL fusion protein is commonly found in infant acute leukaemias of both the myeloid and lymphoid lineage. In order to study the molecular mechanism of haematopoietic progenitor cell (HPC) immortalisation by MLL-ENL, a conditional system of MLL-ENL expression was established in primary murine HPCs. This was achieved by delivering the Tet-Off inducible expression system to primary cells using two retroviral expression constructs. Several conditional immortalised myeloid cell lines were generated *in vitro* which were dependent on continued MLL-ENL expression for their survival and proliferation. The immortalised cells either terminally differentiated or died when MLL-ENL expression was turned off with doxycycline. Since several *Hox* genes are targets of MLL, the expression profile of all 39 murine *Hox* genes was analysed in the MLL-ENL immortalised cell lines by real-time quantitative PCR. Loss of MLL-ENL expression resulted in a decrease in the expression of multiple *Hoxa* genes. By comparing these changes with *Hox* gene expression in cells induced to differentiate with granulocyte-colony-stimulating factor, we found that reduced *Hox* expression was specific to loss of MLL-ENL expression and was not a consequence of differentiation. Affymetrix microarray analysis revealed that MLL-ENL may maintain or activate the expression of the transcription factor Sdccag33 and the serine / threonine kinase Pim-2, which confers protection from apoptosis. The analysis also demonstrated that MLL-ENL may repress the expression of apoptosis promoting genes such as *Ddb2* and *Aatk*. In summary, MLL-ENL is required to initiate and maintain the immortalisation of myeloid progenitors and may contribute to immortalisation by aberrantly maintaining the expression of multiple *Hoxa* genes. The pathways regulated by multiple *Hoxa* genes and the MLL-ENL target genes identified by Affymetrix analysis represent new possibilities for therapies which may combat these aggressive leukaemias.

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This thesis is dedicated to my parents, Richard and Angela.

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Abbreviations

2-ME	2-mercaptoethanol
5-FU	5-fluorouracil
ABL	acute biphenotypic leukaemia
AGM	aorta-gonad-mesonephrous
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
APC	allophycocyanin
BCR	breakpoint cluster region
BFU-E	burst-forming-unit erythrocyte
cDNA	complimentary DNA
CFU	colony forming-unit
CFU-GEMM	CFU-granulocyte-erythrocyte-macrophage-megakaryocyte
CFU-GM	CFU-granulocyte-macrophage
CFU-M	CFU-macrophage
cRNA	complimentary RNA
CLP	common lymphoid progenitor
CML	chronic myeloid leukaemia
CMP	common myeloid progenitor
CMV	Cytomegalovirus
CpG	Cytosine- Guanine dinucleotide
CSF	colony stimulating factor
CSFR	colony stimulating factor receptor
C_T	cycle threshold
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dox	doxycycline
DTT	dithiothreitol
E12	embryonic day 12
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra acetic acid
EGFP	enhanced green fluorescent protein
ES	embryonic stem
EST	expressed sequence tag
FACS	fluorescence activated cell sorting
FAM	6-carboxyfluorescein
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Flt-3L	*fms*-like tyrosine kinase 3 ligand
G-CSF	granulocyte CSF
GM-CSF	granulocyte-macrophage CSF
GMP	granulocyte-monocyte progenitor
HA	haemagglutinin tag
HBSS	Hanks balanced saline solution
HDAC	histone deacetylase
HPC	haematopoietic progenitor cell
HRP	horse radish peroxidase

HSC	haematopoietic stem cell
IL	interleukin
IMDM	Iscoves modified Dulbecco medium
INT	p-iodonitrotetrazolium
IRES	internal ribosome entry site
IVT	*in vitro* transcription
kb	kilobases
kDa	kilo Daltons
LMPP	lymphoid primed multipotent progenitor
LSC	leukaemic stem cell
LT-HSC	long-term HSC
LT-LSC	long-term LSC
LTR	long terminal repeat
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MCS	multiple cloning site
MCSF	macrophage-CSF
MEM	minimal essential-apha medium
MEP	megakaryocyte-erythrocyte progenitor
MFI	mean fluorescence intensity
MGG	may grunwald giemsa
MMLV	Moloney murine leukaemia virus
MPP	multi-potent progenitor
mRNA	messenger RNA
MSCV	Murine stem cell virus
MT	methyltransferase
NHEJ	non-homologous end joining
NK	natural killer
NOD/SCID	non-obese diabetic / severe combined immunodeficient
NP-40	nonidet P40
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHD	plant homeodomain
PGK	phosphoglycerate kinase promoter
P-Sp	para-aortic splanchnopleura
PVDF	polyvinylidene fluoride
Q-PCR	quantitative real-time PCR
RIPA	radioimmunoprecipitation assay
Rn	reaction value
RNA	ribonucleic acid
RT-PCR	reverse-transcription PCR
rtTA	reverse tetracycline transactivator
SCF	stem cell factor
SD	standard deviation
SIN	self-inactivating
SDS	sodium dodecyl sulphate
SNL	sub-nuclear localisation
SSC	sodium chloride-sodium citratein
ST-HSC	short-term HSC

ST-LSC	short-term LSC
SV40	Simian virus 40
t-AML	therapy related AML
TAMRA	6-carboxy-tetramethylrhodamine
TE	Tris-EDTA
TetR	tetracycline repressor
topo II	topoisomerase II
TRE	tetracycline response element
tTA	tetracycline transactivator
tTS	tetracycline suppressor
UNG	uracil-N-glycosylase
VP16	Virion protein 16

Chapter 1 Introduction

1.1 Haematopoiesis

Haematopoiesis is the highly ordered process by which all mature blood cells are generated from a small subset of haematopoietic stem cells (HSCs). In the mouse, primitive haematopoiesis begins in the blood islands of the extraembryonic yolk sac on embryonic day 7 (E7) (Moore and Metcalf, 1970). The onset of circulation at around E8.5 coincides with the development of an intraembryonic site of haematopoiesis in the para-aortic splanchnopleura (P-Sp) which develops into the aorta-gonad-mesonephrous (AGM) region (Zon, 1995; Lensch and Daley, 2004). Definitive haematopoiesis commences at around E10 when embryonic HSCs migrate to and colonise the foetal liver (Johnson and Moore, 1975). However, it remains uncertain as to whether the definitive HSC precursors are derived from the yolk sac (Yoder *et al.*, 1997) or the AGM (Muller *et al.*, 1994; Medvinsky and Dzierzak, 1996). Late in gestation, definitive HSCs migrate from the foetal liver to the bone marrow where they remain throughout adult life (Keller *et al.*, 1999).

HSCs possess the ability to self-renew and differentiate into all haematopoietic lineages. This differentiation programme involves the step-wise production of lineage-restricted progeny with progressively less potential to self-renew. Long-term HSCs (LT-HSCs) give rise to short-term HSCs (ST-HSCs) with limited self-renewal ability. Until recently, the earliest lineage restriction step was thought to involve the commitment of a ST-HSC to either the lymphoid or myeloid differentiation pathways resulting in the generation of either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP). The CMP gives rise to either granulocyte-monocyte progenitors (GMPs) which in turn generate mature granulocytes and monocytes, or megakaryocyte-erythrocyte progenitors (MEPs) which give rise to erythrocytes and platelet-producing megakaryocytes (Akashi *et al.*, 2000). The CLP predominantly gives rise to natural killer (NK) cells and both B and T lymphocytes (Kondo *et al.*, 1997). This traditional model of haematopoiesis is summarised in Figure 1.1A.

Foetal CMPs are not entirely restricted to the myeloid lineage since they also possess limited B cell differentiation potential (Traver *et al.*, 2001). Likewise, foetal

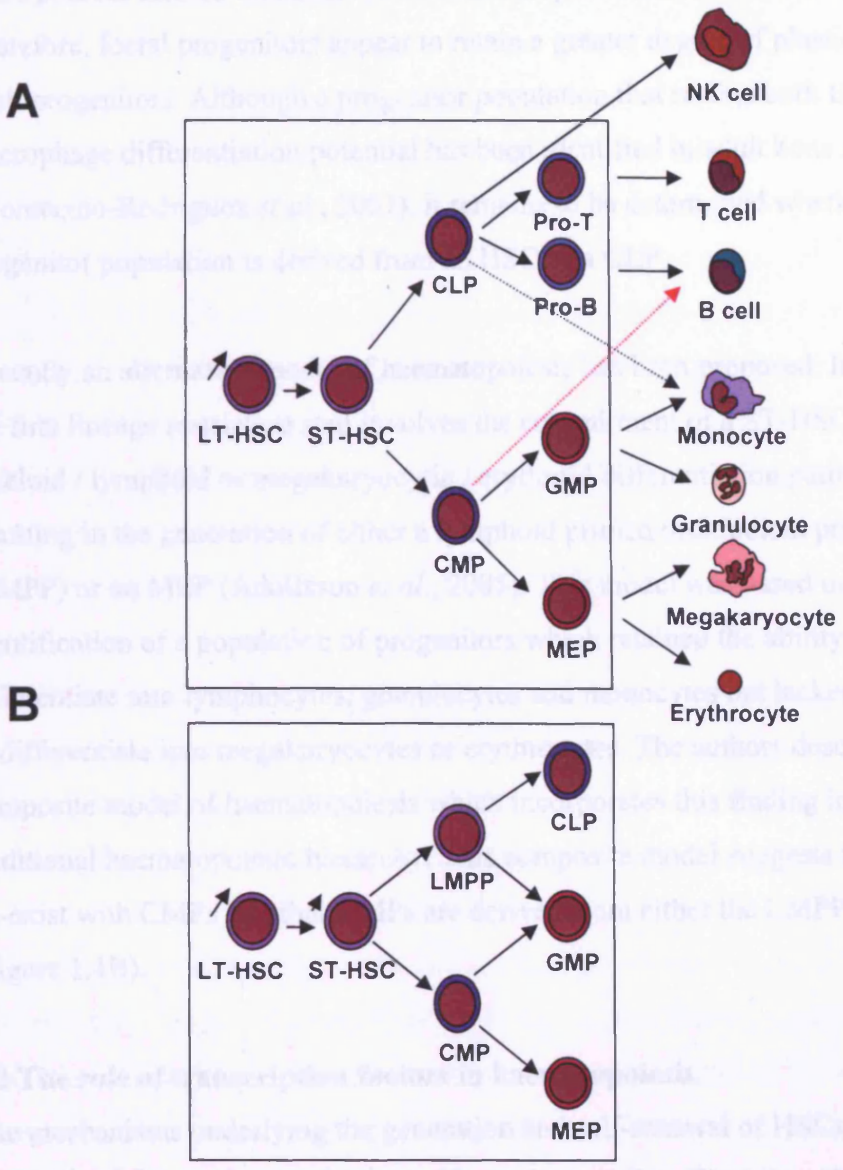

Figure 1.1 Maturation pathways of haematopoietic cells from HSCs. A) The traditional model of haematopoiesis. Long term haematopoietic stem cells (LT-HSCs) with the potential to continuously self-renew, give rise to short-term HSCs (ST-HSCs) with limited self-renewal ability. ST-HSCs give rise to the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) which in turn generate all mature cell types of the lymphoid and myeloid lineages respectively. CLPs exhibit limited monocytic differentiation potential shown by the dashed line. Foetal CMPs are not completely restricted to the myeloid lineage since they are also capable of generating B cells as shown by the red dashed line Adapted from Traver *et al.*, 2001. **B)** The composite model of early haematopoiesis. In this model, ST-HSCs give rise to the lymphoid primed multi-potent progenitor (LMPP) and the CMP. LMPPs then give rise to CLPs and GMPs. The terminal differentiation pathways of the CLP, CMP, GMP and MEP progenitors are identical to that outlined in **A**. Pro-T: T cell progenitor, pro-B: B cell progenitor, NK: natural killer cell, GMP: granulocyte-monocyte progenitor, MEP: megakaryocyte-erythrocyte progenitor. Adapted from Adolfsson *et al.*, 2005.

CLPs possess limited monocyte differentiation potential (Mebius *et al.*, 2001). Therefore, foetal progenitors appear to retain a greater degree of plasticity than adult progenitors. Although a progenitor population that retains both B cell and macrophage differentiation potential has been identified in adult bone marrow (Montecino-Rodriguez *et al.*, 2001), it remains to be determined whether this progenitor population is derived from an HSC or a CLP.

Recently an alternative model of haematopoiesis has been proposed. In this model the first lineage restriction step involves the commitment of a ST-HSC to either the myeloid / lymphoid or megakaryocytic / erythroid differentiation pathways resulting in the generation of either a lymphoid primed multipotent progenitor (LMPP) or an MEP (Adolfsson *et al.*, 2005). This model was based on the identification of a population of progenitors which retained the ability to differentiate into lymphocytes, granulocytes and monocytes but lacked the potential to differentiate into megakaryocytes or erythrocytes. The authors describe a composite model of haematopoiesis which incorporates this finding into the traditional haematopoietic hierarchy. This composite model suggests that LMPPs co-exist with CMPs and that GMPs are derived from either the LMPP or the CMP (Figure 1.1B).

1.2 The role of transcription factors in haematopoiesis.

The mechanisms underlying the generation and self-renewal of HSCs and their commitment to various lineages are extremely complex. Gene targeting studies in mice have revealed specific roles for various transcription factors in the regulation of haematopoiesis. The Stem Cell Leukaemia haematopoietic transcription factor (SCL) and LIM domain only 2 (Lmo2) are essential for both primitive and definitive haematopoiesis (Warren *et al.*, 1994; Robb *et al.*, 1995; Shivdasani *et al.*, 1995). In contrast, Acute Myeloid Leukaemia 1 (AML-1) is dispensable for primitive haematopoiesis but is essential for definitive haematopoiesis (Okuda *et al.*, 1996). Interestingly, a conditional gene targeting strategy revealed that while SCL was absolutely required for the generation of HSCs, it was not required for adult HSC function (Mikkola *et al.*, 2003). A similar approach revealed that AML-1 was also dispensable for adult HSC function (Ichikawa *et al.*, 2004). Therefore, although these transcription factors are essential for the generation and localisation

of HSCs in the embryo (Cai *et al.*, 2000), other factors regulate the functions of mature HSCs.

Other transcription factors such as GATA-1, C/EBPα and Pax5 are essential for the development of specific haematopoietic lineages. PU.1 is essential for both myeloid and lymphoid development (Scott *et al.*, 1994; McKercher *et al.*, 1996). GATA-1 and FOG-1 are required for the development of the erythrocyte and megakaryocyte lineages (Pevny *et al.*, 1991; Shivdasani *et al.*, 1997; Tsang *et al.*, 1998) and C/EBPα is essential for the development of the neutrophil lineage (Zhang *et al.*, 1997). Pax5 and E2A are required for commitment to and development of the B cell lineage (Urbanek *et al.*, 1994; Zhuang *et al.*, 1994; Nutt *et al.*, 1999) and GATA-3 is required for T cell development (Ting *et al.*, 1996).

Multi-potent haematopoietic progenitors (MPPs) exhibit a promiscuous gene expression profile since they co-express low levels of lymphoid, myeloid and erythroid specific genes (Akashi *et al.*, 2003). This finding suggests that these progenitors preserve their multipotent differentiation capacity by maintaining an open chromatin structure which allows transcriptional accessibility to multiple differentiation programmes (Cross and Enver, 1997; Akashi *et al.*, 2003). Lineage commitment results in the amplification of the transcriptional programme specific to that lineage and the subsequent repression of transcriptional programmes associated with other lineages (Enver and Greaves, 1998). For example, GATA-1 and PU.1 are co-expressed in CMPs. The commitment of a CMP to a GMP is associated with an increase in PU.1 expression and a decrease in GATA-1 expression (Zhu and Emerson, 2002). Interestingly, PU.1 is able to bind directly to GATA-1 and this interaction results in an inhibition of DNA binding by GATA-1 (Zhang *et al.*, 1999; Zhang *et al.*, 2000). Therefore, PU.1 permits granulocyte / monocyte development by activating the expression of genes specific to these lineages and directly repressing the erythrocyte / megakaryocyte transcriptional programme by inhibiting GATA-1 function.

1.3 Human acute leukaemia

Acute leukaemia is characterised by a clonal expansion of myeloid blasts (acute myeloid leukaemia) or lymphoid blasts (acute lymphoblastic leukaemia) in the bone

marrow or blood (Downing and Shannon, 2002). Although carcinogenesis is usually a multi-step process in which multiple genetic aberrations culminate in tumour formation, leukaemogenesis may result from as little as two genetic changes (Knudson, 1992). One genetic change might even be sufficient for leukaemogenesis if the mutation affects multiple pathways. A current hypothesis is that a mutation which impairs or blocks differentiation, when combined with a mutation that promotes proliferation or blocks apoptosis, is sufficient for leukaemogenesis (Dash and Gilliland, 2001). The study of paediatric lymphoid leukaemias with the t(12;21) chromosomal translocation, which generates the TEL-AML1 fusion protein, have revealed that this chromosomal translocation is the primary genetic event and that it generates a pre-leukaemic clone which is clinically covert. The acquisition of secondary genetic changes by the pre-leukaemic clone such as loss of the remaining *TEL* allele may then result in leukaemogenesis (Greaves, 1999).

Chromosomal translocations involving genes encoding transcription factors are observed in as many as 65% of cases of acute leukaemia (Look, 1997). Chromosomal translocations de-regulate normal expression of the transcription factor by one of two mechanisms. The transcription factor gene can undergo translocation into regions of genes that are highly transcriptionally active, e.g. translocation of the *myc* gene into the immunoglobulin locus of B cell progenitors is associated with B-cell leukaemia and Burkitt's lymphoma. Alternatively, the transcription factor undergoes a non-random reciprocal chromosomal translocation with a particular partner gene to generate a novel in-frame chimeric fusion gene with altered properties. Examples include *TEL-AML* and *E2A-HLF*, both of which are associated with lymphoid leukaemia (Look, 1997).

1.4 *MLL*-translocations in acute leukaemia

Translocations involving the *MLL* (Mixed Lineage Leukaemia) gene on chromosome band 11q23 are associated with leukaemias of both the myeloid and lymphoid lineage. *MLL* re-arrangements are present in approximately 7-10% of cases of acute lymphoblastic leukaemia (ALL) and 5-6% of cases of acute myeloid leukaemia (AML) (Erfurth *et al.*, 2004). They are most common in infant leukaemia where they comprise 80% of ALL cases and 60% of AML cases (Pui *et al.*, 1995). They are also prevalent in treatment-related secondary leukaemias

(Felix, 1998). Tandem duplications of the *MLL* gene or deletions of *MLL* exon 8 are found in a small subset of leukaemias (Schichman *et al.*, 1995; Caligiuri *et al.*, 1996). However, in the vast majority of cases, *MLL* is joined to a partner gene resulting in the creation of an in-frame chimeric fusion gene. Over 40 fusion partners of *MLL* have now been identified, of which the most common are *AF4*, *ENL* and *AF9* (Scandura *et al.*, 2002). The [t(4;11)(q21;q23)] translocation, which generates the *MLL-AF4* fusion gene, is prevalent in infant pro-B ALL and is associated with a particularly poor prognosis (Heerema *et al.*, 1999). The [t(9;11)(p22;q23)] translocation, which generates the *MLL-AF9* fusion gene, is predominantly associated with AML and the [t(11;19)(q23;p13.3)] translocation, which generates the *MLL-ENL* fusion gene, is associated with both AML and ALL (Rubnitz *et al.*, 1996). The ALL cases associated with *MLL-ENL* and *MLL-AF4* fusion genes usually exhibit a mixed lineage phenotype. The mixed lineage leukaemias are classified as ALL since the blasts are morphologically lymphoid. However, the blasts usually lack expression of the B cell marker CD10 and express myeloid antigens (Pui *et al.*, 1991).

1.5 The generation of *MLL* translocations

Reciprocal translocations involving the *MLL* gene result in the fusion of 5' sequences of the *MLL* gene to 3' sequences of its partner gene. The generation of an *MLL*-translocation involves the cleavage of *MLL* in a localised 8.3 kb region spanning exons 5 to 11 known as the breakpoint-cluster-region (BCR) (Gu *et al.*, 1994). This region of *MLL* is thought to be susceptible to DNA double strand cleavage in response to topoisomerase II (topo II) inhibitors and apoptotic nucleases (Stanulla *et al.*, 1997; Strissel *et al.*, 1998). A current hypothesis is that apoptotic nucleases, which are activated upon the initiation of the apoptotic programme, generate double strand breaks in the *MLL* gene and its prospective partner gene. The two genes are then subsequently joined together by the error-prone non-homologous end joining (NHEJ) DNA repair pathway, resulting in the formation of an *MLL*-translocation (Reichel *et al.*, 1998; Gillert *et al.*, 1999; Betti *et al.*, 2001). While it is generally accepted that the NHEJ pathway plays a role in *MLL*-fusion gene formation, the hypothesis that apoptotic nucleases are responsible for the double strand breaks is controversial since it assumes that cells committed to

undergo apoptosis can somehow circumvent this programme and survive (Betti *et al.*, 2003).

An alternative hypothesis is that topo II inhibitors are responsible for the double strand breaks which lead to the formation of *MLL*-fusion genes. This hypothesis is supported by the finding that patients who develop therapy related AML (t-AML) following treatment of a primary malignancy with topo II inhibitors such as etoposide, often display *MLL*-translocations (Super *et al.*, 1993). Interestingly, the *MLL* breakpoints of infant ALL patients with t(4:11) translocations and of t-AML patients are usually clustered toward the telomeric 3' half of the BCR. In contrast, the majority of *MLL* breakpoints of non-infant ALL patients with the t(4;11) translocation and other *de novo* leukaemias with *MLL*-rearrangements, are clustered towards the cetromeric 5' half of the BCR (Broeker *et al.*, 1996; Cimino *et al.*, 1997; Reichel *et al.*, 2001). This observation suggests that the mechanism underlying *MLL* rearrangements is similar for both infant leukaemias and therapy related leukaemias (Mitterbauer-Hohendanner and Mannhalter, 2004). The high concordance of leukaemia in identical infant twins has led to the theory that the *MLL*-translocation occurs *in utero* (Greaves, 1999). Hence, exposure of the mother to naturally occurring topo II inhibitors such as dietary bioflavonoids may result in the formation of an *MLL*-translocation in the foetus which would then be predisposed to develop leukaemia (Ross *et al.*, 1994; Strick *et al.*, 2000).

1.6 The cell of origin in *MLL*-rearranged leukaemias

Different MLL fusion proteins are associated with leukaemias of different lineages, for example, MLL-AF9 is predominantly associated with myeloid leukaemias whereas MLL-AF4 is found almost exclusively in lymphoid leukaemias. Two models have been proposed which may account for this observation. The instructive model proposes that the *MLL*-translocation occurs in an HSC or uncommitted progenitor and that the MLL fusion partner dictates the lineage of the resultant leukaemia. The non-instructive model is based on the hypothesis that certain translocations are only permissive in particular progenitors. For example, the chromatin configuration of the *AF9* gene may differ between B-cell and myeloid progenitors such that an *MLL-AF9* translocation cannot occur in B cell progenitors but can occur in myeloid progenitors (Daser and Rabbitts, 2004). Alternatively, the

action of the fusion protein may only be permissive in particular lineages. For example the MLL-AF9 fusion protein may only be oncogenic in myeloid progenitors and the MLL-AF4 fusion protein may only be oncogenic in lymphoid progenitors.

There is accumulating evidence which suggests that HSCs are the targets of transformation in AML. The vast majority of human AML cells lack any detectable self-renewal or proliferative capacity (Moore *et al.*, 1973; Buick *et al.*, 1979) and only a very small proportion of these cells were able to engraft and regenerate the leukaemia in NOD/SCID mice (Bonnet and Dick, 1997). This small population of AML cells that were able to regenerate leukaemia in mice were termed leukaemic stem cells (LSCs). LSCs were isolated based on their $CD34^+$ $CD38^-$ immunophenotype, which is shared by normal HSCs (Bonnet and Dick, 1997). Further studies revealed that LSCs, like normal HSCs, are functionally heterogeneous with some possessing extensive self-renewal ability (LT-LSC) and others possessing limited self-renewal ability (ST-LSC) (Hope *et al.*, 2004). The fact that the leukaemias initiated by the LSCs in the NOD/SCID mice were mostly composed of blasts with limited self-renewal and proliferative capacity suggests that AML is arranged as a hierarchy. As with normal HSCs, LSCs can self-renew and give rise to myeloid progeny. However, the myeloid progeny either inherit or acquire mutations that render them unable to terminally differentiate (Warner *et al.*, 2004).

While the studies outlined above provide compelling evidence that HSCs are the targets of transformation in AML, there is also evidence that more committed progenitors such as the CMP and GMP may be targets of transformation in some cases. The retroviral transduction of purified populations of murine HSCs, CMPs and GMPs with MLL-ENL yielded immortalised cell lines (Cozzio *et al.*, 2003). Furthermore, MLL-ENL transduced HSC, CMP and GMP induced leukaemia in recipient mice with similar phenotypes and latencies. However, limiting dilution analysis revealed that MLL-ENL transformed HSCs more efficiently that CMPs or GMPs (Cozzio *et al.*, 2003). Although MLL-ENL translocations are associated with myeloid and lymphoid leukaemias in patients, MLL-ENL was not able to transform a purified population of CLPs (Cozzio *et al.*, 2003). The same was true for MLL-

GAS7 which gave rise to biphenotypic, myeloid or lymphoid leukaemias upon the transduction of a purified HSC population (So *et al.*, 2003a). Therefore, the cell of origin in biphenotypic or mixed lineage leukaemias is most likely an HSC or a bi-potential B/macrophage progenitor.

Similar experiments revealed that although the fusion proteins MOZ-TIF2 and BCR-ABL were both able to transform HSCs, only MOZ-TIF2 was able to transform the CMP and GMP progenitor populations (Huntly *et al.*, 2004). Since MLL-ENL and MOZ-TIF2 are able to transform CMPs and GMPs which lack inherent self-renewal capacity, these fusion proteins must confer self-renewal capability to these progenitors. MLL-ENL and MOZ-TIF2 are both associated with acute myeloid leukaemia in patients whereas BCR-ABL is associated with chronic myeloid leukaemia (CML) and to a lesser extent acute lymphoid leukaemia. Further experiments are required in order to ascertain whether the acute and chronic phases of myeloid leukaemia are distinguished by the ability of the leukaemia associated oncogene to promote aberrant self-renewal activity (Jamieson *et al.*, 2004).

1.7 The function of MLL

MLL is the human homologue of the *Drosophila trithorax* (*TRX*) gene (Tkachuk *et al.*, 1992). TRX positively regulates homeotic (*HOM-C*) gene expression during development since *TRX* mutants exhibit either loss or abnormal patterns of *HOM-C* gene expression resulting in segment identity defects (Breen and Harte, 1993). Importantly, TRX is responsible for the maintenance but not the initiation of *HOM-C* gene expression. The actions of TRX are opposed by the polycomb-group (Pc-G) proteins, which repress homeotic gene expression. TRX and Pc-G proteins are components of large complexes, which associate with chromatin and regulate homeotic gene expression through epigenetic mechanisms which remain poorly understood (Pirrotta, 1998; Canaani *et al.*, 2004).

The normal function of MLL has been studied in mice using various gene-targeting approaches. One strategy was the targeted disruption of exon 3B using a *LacZ* reporter (Yu *et al.*, 1995). An alternative strategy was the replacement of exons 12-14 with a PGK-neo cassette (Yagi *et al.*, 1998). Homozygous mutant mice died before birth at E10.5 in the former study and between E11.5 and E14.5 in the latter

study. Heterozygous mutant mice possessed homeotic transformations of the axial skeleton and exhibited haematological abnormalities such as anaemia and thrombocytopenia (Yu *et al.*, 1995). Analysis of *Hox* (the mammalian homologues of the *Drosophila HOM-C* genes) gene expression in heterozygous mutant embryos revealed a one-segment posterior shift in *Hoxc8* gene expression (Hanson *et al.*, 1999). Interestingly, embryos deficient for *Bmi-1* (the mammalian homologue of the Pc-G gene *Drosophila Posterior Sex combs*) exhibited a one-segment anterior shift in *Hoxc8* expression. The fact that $MLL^{+/-}$ $Bmi\text{-}1^{-/-}$ double mutant mice exhibit normal patterns of *Hoxc8* expression suggest that like the TRX and Pc-G proteins of Drosophila, MLL and Bmi-1 reciprocally regulate the expression of some *Hox* genes (Hanson *et al.*, 1999). Although *MLL* deficient embryos exhibited normal patterns of *Hoxc8* and *Hoxa7* expression prior to E9 (Yu *et al.*, 1998), expression of these genes were not observed after this time-point. Therefore, MLL functions in a similar way to TRX in that it is required to maintain but not initiate the expression of particular *Hox* genes. Subsequent studies have revealed that in addition to *Hoxc8* and *Hoxa7*, MLL maintains the expression of *Hoxa9*, *Hoxa10*, *Hoxb4*, *Hoxb5*, *Hoxb6*, *Hoxb8*, *Hoxc6* and *Hoxc9* (Hanson *et al.*, 1999; Ernst *et al.*, 2004b).

Haematopoietic progenitors isolated from both the yolk sac (Hess *et al.*, 1997) and foetal liver (Yagi *et al.*, 1998) of *MLL* deficient mice demonstrated a reduced clonogenic capacity *in vitro*. The number and size of colony forming unit macrophages (CFU-M), colony forming unit granulocytes-macrophages (CFU-GM), colony forming unit granulocytes-erythrocytes-macrophages-megakaryocytes (CFU-GEMM) and burst forming unit erythrocytes (BFU-E) were markedly reduced in $MLL^{-/-}$ embryos. However, the cells present in these colonies were identical to those generated from $MLL^{+/+}$ and $MLL^{+/-}$ embryos. These results are consistent with a role for MLL in the haematopoietic progenitor, possibly in proliferation or lineage determination but not in later stages of differentiation (Hess *et al.*, 1997).

MLL deficient mice displayed many defects in non-haematopoietic tissues (Yu *et al.*, 1995; Yu *et al.*, 1998; Yagi *et al.*, 1998). This is because MLL is not only expressed in haematopoietic cells but also in a wide variety of other tissues (Butler *et al.*, 1997; Kawagoe *et al.*, 1999). In order to investigate the haematopoiesis

specific functions of MLL, blastocysts were reconstituted with *MLL*-deficient or heterozygous ES cells (Ernst *et al.*, 2004a). Analysis of E14 foetal livers from $MLL^{+/-}$ and $MLL^{-/-}$ chimeras revealed that *MLL*-deficient cells did not contribute to the c-Kit^+ lin^- progenitor population. Although normal numbers of c-Kit^+ cells were present in the AGM of $MLL^{-/-}$ embryos, these cells exhibited reduced expression of many other HSC markers including CD34, CD45 and PECAM. Furthermore E11.5 AGM cells derived from $MLL^{-/-}$ mice failed to reconstitute $RAG\gamma C^{-/-}$ recipients. Surprisingly, detectable donor contribution was only observed in 2 out of 9 recipients engrafted with $MLL^{+/-}$ AGM cells. This data shows that MLL is essential for definitive haematopoiesis. Either MLL is required to generate normal HSCs in the AGM or it is required for HSCs to migrate from the AGM and seed the foetal liver (Ernst *et al.*, 2004a).

Analysis of embryoid bodies which were formed from $MLL^{-/-}$ ES cells revealed that MLL was not required for the development of the hemangioblast (the common mesodermal precursor of HSCs and endothelial cells) (Ernst *et al.*, 2004b). $MLL^{-/-}$ embryoid bodies were able to generate definitive c-Kit^+ $CD41^+$ haematopoietic cells efficiently. However, these cells were unable to produce haematopoietic colonies (Ernst *et al.*, 2004b). Therefore, although MLL is not required for the commitment of mesodermal cells to the haematopoietic lineage, it is absolutely required for the proliferation and subsequent differentiation of haematopoietic cells (Ernst *et al.*, 2004a; Ernst *et al.*, 2004b).

1.8 The role of *Hox* genes in haematopoiesis

The under-expression of a subset of *Hox* genes in *MLL* deficient mice may contribute to their severe haematopoietic defect. Mammalian *Hox (Homeobox)* genes are a family of transcription factors characterised by the presence of a highly conserved 183 nucleotide sequence termed the homeobox which encodes a helix-turn-helix DNA binding domain (Gehring *et al.*, 1994). Mammals possess 39 *Hox* genes, which are organised into four clusters (A, B, C and D), on four different chromosomes (Boncinelli *et al.*, 1989). Each cluster contains between 9 and 11 *Hox* genes which have been assigned to 13 paralog groups. For example, paralog group 1 consists of *Hoxa1*, *Hoxb1* and *Hoxd1*. The genomic organisation of the *Hox* genes is summarized in Figure 1.2.

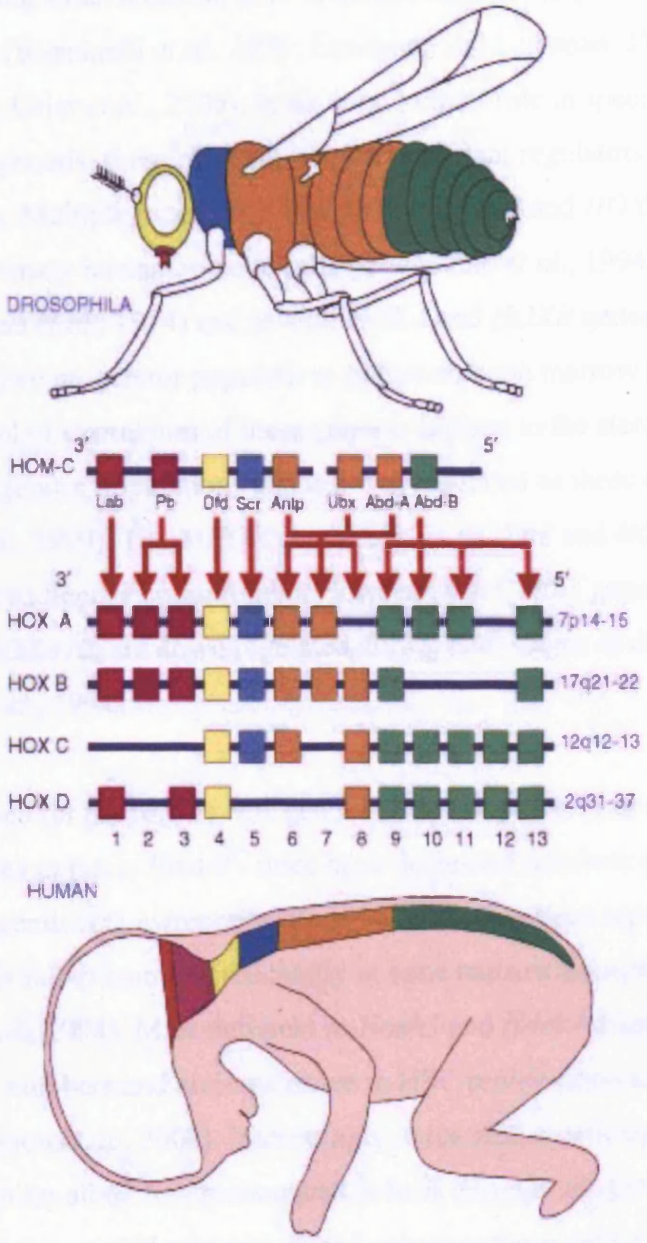

Figure 1.2 The organisation and conservation of the *HOM-C* and *HOX* gene families. In mammals, the four *HOX* gene clusters (A, B C and D) which are organised on four different chromosomes, are thought to have arisen by duplication and divergence. Each cluster contains between 9 and 11 *HOX* genes which have been assigned to 13 paralog groups. During embryogenesis, *HOX* genes specify positional identity along the anterior-posterior axis. Their expression is spatially and temporally regulated such that the 3' *HOX* genes such as *HOXA1* are expressed earlier and in a more anterior position in the embryo than the 5' *HOX* genes such as *HOXA13*. Reproduced from Grier *et al.*, 2005.

Hox proteins are key regulators of the mammalian body plan and play important roles in regulating axial-skeleton, hind-brain and limb development during embryogenesis (Boncinelli *et al.*, 1989; Lawrence and Largman, 1992; Kmita and Duboule, 2003; Grier *et al.*, 2005). In addition to their role in specifying cell fate during embryogenesis, these proteins are also important regulators of haematopoiesis. Multiple members of the *HOXA*, *HOXB* and *HOXC* clusters are expressed in primary haematopoietic cells (Giampaolo *et al.*, 1994; Moretti *et al.*, 1994; Sauvageau *et al.*, 1994) and several *HOXA* and *HOXB* genes are expressed in the most primitive progenitor populations of human bone marrow (Sauvageau *et al.*, 1994). The level of expression of these genes is highest in the stem cell and committed progenitor populations and is down-regulated as these cells differentiate (Kawagoe *et al.*, 1999). The 3'*HOX* genes such as *HOXB3* and *HOXB4* are down regulated prior to lineage commitment. However, the 5'*HOX* genes, such as *HOXA9* and *HOXA10*, are down regulated during later stages of differentiation (Sauvageau *et al.*, 1994).

Further evidence for the role of *Hox* genes in haematopoiesis has come from gene targeting studies in mice. *Hoxa9*$^{-/-}$ mice have decreased numbers of myeloid and pre-B cell progenitors (Lawrence *et al.*, 1997) and it has been reported that *Hoxa9* deficient HSCs fail to compete efficiently in bone marrow transplantation chimeras (Lawrence *et al.*, 1998). Mice deficient in *Hoxb3* and *Hoxb4* displayed reductions in foetal HSC numbers and an impairment in HSC proliferation and repopulating ability (Bjornsson *et al.*, 2003). Interestingly, mice with a deficiency in *Hoxb4* alone have similar albeit less pronounced defects (Brun *et al.*, 2004). Therefore, the combined deficiency of *Hoxb4* and *Hoxb3* enhances these defects, without altering the phenotype, in a dose-dependent manner.

Mice engineered to over-express certain *Hox* genes by retroviral transduction, followed by bone marrow transplantation, have provided further insight into the role of these genes in haematopoiesis. Mice over-expressing *Hoxa9* displayed an expansion of HSCs and myeloid progenitor cells (Thorsteinsdottir *et al.*, 2002) and eventually developed AML (Kroon *et al.*, 1998). Over-expression of *Hoxa10* conferred a proliferative advantage to myeloid progenitors (Bjornsson *et al.*, 2001), promoted megakaryocytic differentiation at the expense of macrophage

differentiation and induced AML (Thorsteinsdottir *et al.*, 1997). Over-expression of *Hoxa10*, like *Hoxa9*, caused a block in B cell development at the pre-B cell progenitor stage suggesting that down-regulation of these genes is required for normal B cell development (Thorsteinsdottir *et al.*, 1997; Thorsteinsdottir *et al.*, 2002). Over-expression of *Hoxb3* caused a block in both B cell and T cell development and induced a myeloproliferative disorder which progressed to AML (Sauvageau *et al.*, 1997). In contrast, over-expression of *Hoxb4* enhanced HSC proliferation and self-renewal without altering differentiation or promoting leukaemic development (Sauvageau *et al.*, 1995). Interestingly, Hoxa9, Hoxa10 or Hoxb4 were able to rescue haematopoietic colony formation from $MLL^{-/-}$ embryoid bodies, although the subsequent differentiation of the colonies varied according to the particular *Hox* gene expressed (Ernst *et al.*, 2004b). This finding suggests that although Hoxa9, Hoxa10 and Hoxb4 promote the development of different haematopoietic lineages, they are functionally redundant in their ability to promote the proliferation of immature haematopoietic cells (Ernst *et al.*, 2004b).

1.9 The functional domains of MLL

The *MLL* gene spans 90 kb, possesses 37 exons and encodes a 431 kDa protein, which is proteolytically cleaved into two fragments. MLL is cleaved at two conserved sites by a novel protease called Taspase 1 (Hsieh *et al.*, 2003b). This cleavage yields an N-terminal 320 kDa fragment and a C-terminal 180 kDa fragment. These two fragments heterodimerise and their interaction confers stability and correct subnuclear localisation of the protein (Yokoyama *et al.*, 2002; Hsieh *et al.*, 2003a; Hsieh *et al.*, 2003b). Several domains of MLL are now well characterised. These include the AT hooks, the DNA methyltransferase homology domain and the SET domain. The domain structure of the full-length MLL protein, the proteolysis and subsequent interaction of the N and C-terminal fragments are depicted in Figure 1.3.

Sub-nuclear localisation domains

The sub-nuclear localisation domains (SNL1 and SNL2) are responsible for determining the punctate expression pattern of MLL in the nucleus (Yano *et al.*, 1997).

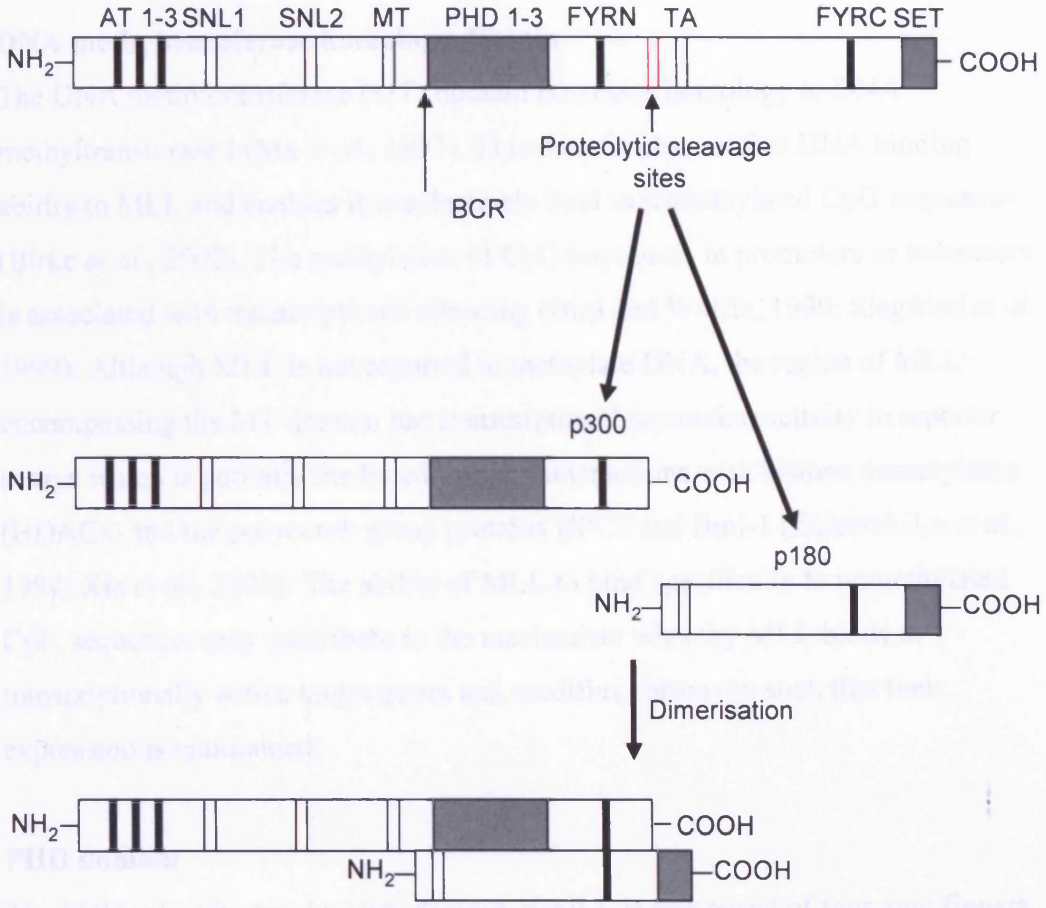

Figure 1.3 Structure of the wild-type MLL protein. MLL is proteolytically cleaved by taspase (a threonine aspartase) at two conserved sites near its C-terminus. Cleavage results in the generation of a large N-terminal peptide of 300kDa (p300) and a smaller C-terminal peptide of 180kDa (p180) which interact *in vivo* via the FYRN domain of p300 and the FYRC domain of p180. AT1-3: AT hooks, SNL1-2: sub-nuclear localisation domains, MT: methyltransferase homology domain, PHD1-3: PHD fingers, TA: transactivation domain. Adapted from Eguchi *et al.*, 2003.

AT hooks

MLL contains three closely spaced AT hooks which enable MLL to bind to AT-rich regions of DNA in the minor groove. However, they recognise DNA structure (intrinsically bent or cruciform DNA) rather than specific sequences (Aravind and Landsman, 1998).

DNA methyltransferase homology domain

The DNA methyltransferase (MT) domain possesses homology to DNA methyltransferase I (Ma *et al.*, 1993). This domain also confers DNA binding ability to MLL and enables it to selectively bind to unmethylated CpG sequences (Birke *et al.*, 2002). The methylation of CpG sequences in promoters or enhancers is associated with transcriptional silencing (Bird and Wolffe, 1999; Siegfried *et al.*, 1999). Although MLL is not reported to methylate DNA, the region of MLL encompassing the MT domain has transcriptional repression activity in reporter assays which is partially mediated through interactions with histone deacetylases (HDACs) and the polycomb group proteins HPC2 and Bmi-1 (Zeleznik-Le *et al.*, 1994; Xia *et al.*, 2003). The ability of MLL to bind specifically to unmethylated CpG sequences may contribute to the mechanism whereby MLL binds to transcriptionally active target genes and modifies chromatin such that their expression is maintained.

PHD domain

The PHD (plant homeodomain) domain of MLL is composed of four zinc fingers which are highly conserved with TRX. PHD fingers are present in many proteins that are involved in chromatin-associated transcriptional regulation (Aasland *et al.*, 1995). Therefore, it is considered that the PHD domain of MLL may play an important role in regulating the expression of MLL target genes. The PHD zinc fingers of MLL can homodimerise and the third zinc finger is able to bind to the nuclear cyclophilin Cyp33 (Fair *et al.*, 2001). Interestingly, the binding of Cyp33 to the PHD domain increased the binding of HDAC1 to the repression domain of MLL (Xia *et al.*, 2003). Furthermore, the over-expression of Cyp33 in cells containing wild-type MLL resulted in the down-regulation of the MLL target genes *HOXC8* and *HOXC9* (Fair *et al.*, 2001). This down-regulation was dependent upon the cis / trans prolyl-isomerase activity of Cyp33, since it was not observed in the

presence of the cis / trans prolyl-isomerase inhibitor cyclosporine (Fair *et al.*, 2001). This data suggests that the cis / trans prolyl-isomerase activity of Cyp33 alters the conformation of MLL to allow HDAC1 binding and the subsequent repression of MLL target genes (Xia *et al.*, 2003).

Transactivation domain

In addition to the repression domain, a transactivation domain exists between the PHD and SET domains of MLL (Prasad *et al.*, 1995). This transactivation domain facilitates the binding of MLL to the transcriptional co-activator CBP (cAMP binding protein), which promotes its interaction with CREB (cAMP response element binding) and thus enhances transcriptional activation (Ernst *et al.*, 2001). Cleavage of MLL by Taspase 1 at a conserved proteolytic site between the PHD and transactivation domains results in the generation of two peptides, with opposite transcriptional properties. The N-terminal peptide contains the repression domain of MLL whereas the C-terminal peptide contains the transactivation domain of MLL (Figure 1.3). The post-translational processing of MLL by Taspase 1 is therefore a potential mechanism whereby the transcriptional activity of MLL is regulated (Yokoyama *et al.*, 2002).

SET domain

The SET (Su(var)3-9, enhancer of zeste and trithorax) domain of MLL is the most highly conserved domain between MLL and TRX. Previous studies have shown that this domain possesses histone H3 lysine 4-specific methyltransferase activity, which is stimulated by H3 peptides that are acetylated on lysine 9 or lysine 14 (Milne *et al.*, 2002; Nakamura *et al.*, 2002). Since H3-K4 histone methylation is associated with transcriptional activation (Nakamura *et al.*, 2002), it is likely that the SET domain plays an important role in regulating MLL target gene expression. Accordingly, the SET domain of MLL was required for the transcriptional activation of *Hoxc8* upon the re-expression of *MLL* in *MLL* null fibroblasts (Milne *et al.*, 2002). This was mediated by direct binding of MLL to the *Hoxc8* promoter and subsequent H3-K4 methylation of the *Hoxc8* promoter and 5' enhancer. MLL was also able to methylate the promoters of *Hoxa7* and *Hoxa9* (Milne *et al.*, 2002) suggesting that H3-K4 methylation of MLL target gene promoters is likely to be a common mechanism of transcriptional activation by MLL. The re-expression of

MLL in *MLL* null cells also resulted in H3 and H4 acetylation of the 5' and 3' enhancers of *Hoxc8* (Milne *et al.*, 2002). This suggests that MLL also promotes histone acetylation of its target genes. In accordance with this, a recent study demonstrated that MLL was able to interact with the H4-K16 specific histone acetyltransferase MOF (Dou *et al.*, 2005). Importantly, both H3-K4 methylation and H4-K16 acetylation of the *Hoxa9* locus were required for optimal *Hoxa9* expression (Dou *et al.*, 2005). Therefore, MLL recruits coactivators to its target gene promoters, which cooperate with MLL to maintain the expression of MLL target genes.

1.10 MLL is a component of a multi-protein super complex.

It is now understood that MLL is a component of a super-complex which consists of more than 29 proteins, which acetylate, deacetylate and methylate histones (Nakamura *et al.*, 2002). Many of the proteins associated with MLL are components of transcription complexes. These include components of the TFIID complex (which is required for the assembly of the transcriptional preinitiation complex), the SWI/SNF chromatin remodelling complex and the NuRD and Sin3A histone deacetylases complexes. Many of these components were identified at the promoter of *HOXA9* which suggests that these components are required by MLL to remodel the chromatin of MLL target genes and thus maintain their expression (Nakamura *et al.*, 2002).

Recently, another complex of MLL was identified whose components included HCF-2, ASH2L1, menin, RBBP5 and WDR5 (Yokoyama *et al.*, 2004). Of these components only menin, which was originally identified as a product of the MEN1 tumour suppressor gene, was found to be crucial for the maintenance of *HOXA9* expression. Loss of menin expression resulted in a similar decrease in *HOXA9* expression as loss of MLL expression (Yokoyama *et al.*, 2004). Therefore, menin plays an important role in regulating the expression of MLL target genes. This was highlighted by the finding that menin promoted the binding of MLL to target gene promoters (Milne *et al.*, 2005). Although many of the components of this new complex are similar to that of the yeast and human SET1 histone methyltransferase complexes (Miller *et al.*, 2001; Roguev *et al.*, 2001; Nagy *et al.*, 2002; Wysocka *et al.*, 2003), only WDR5 and RBBP5 were present in the previously identified MLL

super-complex (Nakamura *et al.*, 2002). The authors speculated that the composition of the complex may vary with different cellular conditions and this may account for the lack of common components between the two MLL complexes (Yokoyama *et al.*, 2004).

1.11 MLL domains retained in fusion proteins

The AT hooks, sub-nuclear localisation domains and MT homology domain of MLL are consistently retained in the MLL fusion protein whereas the PHD, transactivation and SET domains of MLL are replaced by sequences of the partner protein (Figure 1.4). The MLL fusion protein also lacks the Taspase 1 proteolytic cleavage site and the FYRN domain required for interaction with the p180 C-terminal MLL fragment. Since the binding of Cyp33 to the PHD domain represses MLL target genes, the loss of the PHD domain may convert MLL to a constitutive activator (Fair *et al.*, 2001).

MLL is thought to maintain appropriate expression levels of its target genes, which are required for normal haematopoietic differentiation, through the H3-K4 methyltransferase activity of its SET domain. Since MLL-fusion proteins lack this domain, it is likely that they will not be able to maintain appropriate expression levels of MLL target genes. The stability and transcriptional activity of the MLL fusion protein is expected to differ from the wild-type MLL protein since the fusion protein is not processed by Taspase 1 (Hsieh *et al.*, 2003a). It is likely that the composition of the MLL-fusion protein super complex will also vary from that of wild-type MLL. In support of this, MLL-fusion proteins retain the ability to interact with menin but not other components of the MLL/HCF super complex (Yokoyama *et al.*, 2004).

1.12 Functions of the MLL fusion partners

Some of the MLL fusion partners share homology, for example *AF4/AF5q31/LAF4*, *ENL/AF9* and *AF10/AF17* are members of common gene families (Nakamura *et al.*, 1993; Chaplin *et al.*, 1995; Nilson *et al.*, 1997; Taki *et al.*, 1999). However, most of the fusion partners share no homology or common functional domains. Therefore, they have generally been divided into two categories, signalling molecules that localise to the cytoplasm, or nuclear factors involved in transcriptional regulation

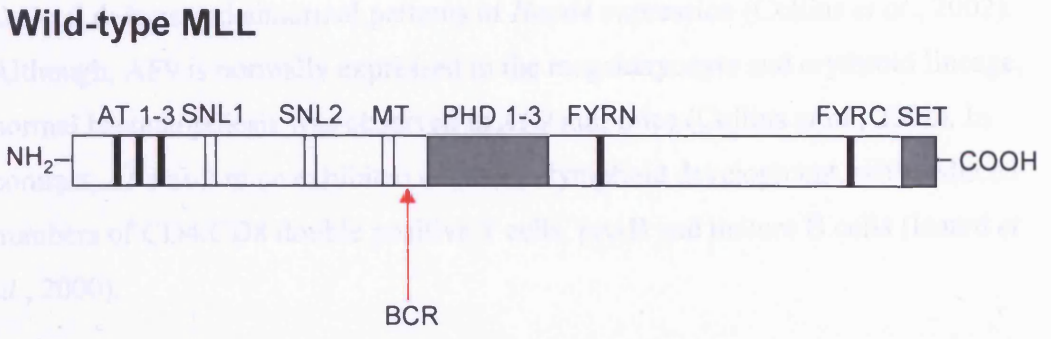

Figure 1.4 Structure of wild-type MLL and a typical MLL-fusion protein. In all *MLL*-fusion genes examined, *MLL* is cleaved within an intron in a conserved region known as the breakpoint-cluster-region (BCR) which spans exons 5-8. Once *MLL* is cleaved, it is joined to the 3' sequences of one of its many partner genes resulting in the generating an in-frame chimeric fusion protein. The AT hooks (AT 1-3), sub-nuclear localisation domains (SNL1 and 2) and the methyltransferase homology (MT) domain of MLL are retained in the fusion protein. In contrast, the plant homeodomain (PHD1-3), the FYRN and FYRC domains which mediate the interactions of the p300 and p180 MLL fragments and the SET domain are replaced by the C-terminal sequences of the partner protein.

(Eguchi *et al.*, 2003). The function of each MLL partner protein and its cellular localisation is summarised in Table 1.1.

AF4, AF9 and ENL are the most common MLL fusion partners and all three possess transcriptional activation function in reporter assays (Rubnitz *et al.*, 1994; Prasad *et al.*, 1995; Morrissey *et al.*, 1997). Like MLL, AF9 is required for embryo patterning and normal *Hox* gene expression since *AF9* null mice possess axial skeletal defects and abnormal patterns of *Hoxd4* expression (Collins *et al.*, 2002). Although, AF9 is normally expressed in the megakaryocyte and erythroid lineage, normal haematopoiesis was observed in *AF9* null mice (Collins *et al.*, 2002). In contrast, *AF4* null mice exhibited defects in lymphoid development, with reduced numbers of CD4/CD8 double positive T cells, pre-B and mature B cells (Isnard *et al.*, 2000).

AF9, ENL and GAS41 share homology to TGF3/ANC1 which binds to the yeast chromatin re-modelling complex SWI/SNF through its interactions with SNF5 (Cairns *et al.*, 1996). Although GAS41 is not a direct fusion partner of MLL, it is able to interact with the MLL fusion partner AF10 (Debernardi *et al.*, 2002). A role for ENL in chromatin remodelling is supported by the finding that it is a component of a novel human SWI/SNF chromatin remodelling complex (Nie *et al.*, 2003). Since ENL and AF9 share significant homology and both interact with the Polycomb 3 (hPc3) protein (Garcia-Cuellar *et al.*, 2001), it is likely that AF9 is also a component of a chromatin remodelling complex. Interestingly, GAS41 has been shown to interact with INI1 (the human homologue of the yeast SWI/SNF component SNF5) and may therefore act as a bridging factor between AF10 and SWI/SNF complexes. More recently, AF10 has been shown to interact with hDOT1L which possesses H3-K79 histone methyltransferase activity (Okada *et al.*, 2005). Hence, like MLL, many of the MLL fusion partners may be either directly or indirectly involved in chromatin remodelling.

Table 1.1 Cloned *MLL*-partner genes and chromosomal abnormalities involving the *MLL* gene on chromosome 11q23.

Gene	Cytogenetic abnormality	Function	Localisation
ABI-1	t(10;11)(p11.2;q23)	Regulator of endocytosis? / cell motility	C
AF1p	t(1;11)(p32;q23)	Regulator of endocytosis	C, N
AF1q	t(1;11)(q21;q23)	NK	NK
AF3p21	t(3;11)(p21;q23)	NK	N
AF4	**t(4;11)(q21;q23)**	**Transcriptional activator**	**N**
AF5q31	t(5;11)(q31;q23)	Homology to AF4, transcriptional activator?	N?
AF6	**t(6;11)(q27;q23)**	**Maintenance of cell-cell junctions and cell polarity**	**C**
AF9	**t(9;11)(q22;q23)**	**Homology to ENL, transcriptional activator**	**N**
AF9q34	t(9;11)(q34;q23)	Ras GTPase-activating protein	C
AF10	**t(10;11)(p12;q23)**	**Transcription factor**	**N**
AF15q14	t(11;15)(q23;q14)	NK	NK
AF17	t(11;17)(q23;q21)	Transcription factor	N
AFX	t(X;11)(q13;q23)	Forkhead transcription factor	N
CALM	inv(11)(q14;q23)	Regulator of endocytosis, clathrin assembly protein	C, N
CBL	del(11)	Negative regulator of receptor tyrosine kinases	C
CBP	t(11;16)(q23;p13)	Transcriptional co-activator, Histone acetylase	N
EEN	t(11;19(p13;q23)	Regulator of endocytosis?	C?
ELL	**t(11;19)(q23;p13.1)**	**Transcriptional activator***	**N**
ENL	**t(11;19)(q23;p13.3)**	**Transcriptional activator**	**N**
FBP17	ins(11;9)(q23;q34) inv(11)(q13)(q23)	NK	C
FKHRL1	t(6;11)(q21;q23)	Forkhead transcription factor	N
GAS7	t(11;17(q23;p13)	Growth-arrest specific gene	C

GMPS	t(3;11)(q25;q23)	Guanosine monophosphate synthetase	C
GPHN	**t(11;14)(q23;q24)**	**Gly and GABA receptor assembly**	**C**
GRAF	t(5;11)(q31;q23)	Negative regulator of RhoA	C
hCDCrel	**t(11;22)(q23;q11.2)**	**Septin family**	**C**
LAF4	t(2;11)(q11.2-12;q23)	Homology to AF4, transcriptional activator	N
LARG	**del(11)**	**Activator of Rho GTPases**	**C**
LASP1	t(11;17)(q23;q21)	NK	C
LCX	**t(10;11)(q22;q23)**	**NK**	**NK**
LPP	t(3;11)(q28;q23)	Regulator of cell motility and focal adhesion	C,N
MPFYVE	**t(11;15)(Q23;q14)**	**NK**	**NK**
MSF	t(11;17)(q23;q25)	Septin family	C
p300	**t(11;22)(q23;q13)**	**Transcriptional co-activator, histone acetylase**	**N**
Septin6	ins(X;11)(q24;q23)	Septin family	C

The most common MLL partner genes are highlighted in bold. N indicates nuclear localisation, C indicates cytoplasmic localisation and NK means not known. * indicates that the transcriptional activation activity is indirect and is mediated through binding to EAF1 (Luo *et al.*, 2001). Adapted from Eguchi *et al.*, 2003 and Mitterbauer-Hohendanner and Mannhalter, 2004.

Many of the fusion partner proteins interact with each other such as AF4 and AF9 (Erfurth *et al.*, 2004) and ENL and ABI1 (Garcia-Cuellar *et al.*, 2000). More recently ENL was found to interact with AF4 and AF5q31 (Zeisig *et al.*, 2005). These interactions have led to the proposal of the 'MLL web hypothesis' which suggests that although the partner proteins do not share homology or a common function they may all be part of a large complex network which is disrupted by the formation of an MLL-fusion protein (Erfurth *et al.*, 2004).

1.13 Models of MLL leukaemias

Various *in vitro* and *in vivo* models have been established in order to investigate whether MLL-fusion proteins are leukaemogenic.

***In vitro* models**

Prior to the development of animal models, the properties of MLL fusion proteins were examined by over-expressing them in haematopoietic cell lines. The murine 32Dc13 myeloid cell line is IL-3 responsive and normally undergoes terminal neutrophil differentiation in response to G-CSF. The over-expression of an N-terminal MLL protein, which was truncated at the BCR, inhibited the differentiation of 32Dc13 cells in response to G-CSF. However, cells over-expressing full-length MLL differentiated normally in response to G-CSF (Joh *et al.*, 1996). This finding suggests that MLL fusion proteins may act in a dominant negative fashion by disrupting the normal function of MLL (Joh *et al.*, 1999). Attempts at generating stable 32Dc13 cell lines over-expressing the MLL-AF9 or MLL-ENL fusion genes failed. Therefore, inducible systems of fusion protein expression were generated. Wild-type 32Dc13 cells or a stable 32Dc13 cell line expressing full-length MLL displayed an up-regulation of *Hoxa7*, *Hoxb7* and *Hoxc9* expression upon G-CSF treatment (Joh *et al.*, 1999). However, the induction of expression of MLL-AF9 or N-terminal truncated MLL inhibited the up-regulation of these *Hox* genes in response to G-CSF (Joh *et al.*, 1999). Truncated MLL may therefore block G-CSF induced differentiation of 32Dc13 cells by disrupting *Hox* gene expression. It was not reported whether MLL-AF9 also blocked the G-CSF induced differentiation of 32Dc13 cells.

The induction of MLL-AF9 expression in the U937 human monoblastic cell line induced growth arrest, macrophage differentiation and apoptosis of these cells (Caslini *et al.*, 2000). Deletion mutagenesis revealed that the N-terminus of MLL spanning the AT hooks was responsible for the induction of growth arrest and differentiation. A more detailed study found that graded expression of MLL-AF9, which was achieved using different concentrations of tetracycline, always resulted in a higher percentage of cells in S-phase than cells which expressed only the N-terminus of MLL (Caslini *et al.*, 2004). This finding suggests that MLL-AF9 may confer a proliferative advantage to the cells even though MLL-AF9 expression is ultimately not compatible with survival in this cell line (Caslini *et al.*, 2004). The induction of MLL-AF4 expression in U937 cells resulted in a decrease in growth rate and partial macrophage differentiation (Caslini *et al.*, 2004). Interestingly, cells expressing MLL-AF4 exhibited an increased survival in response to highly

cytotoxic doses of etoposide. This finding is consistent with the observation that cell lines derived from patients with t(4;11) translocations proliferate more slowly than non-t(4;11) derived cell lines and are resistant to apoptosis induced by serum deprivation (Kersey *et al.*, 1998). The apparent difficulty in generating stable cell lines over-expressing certain MLL-fusion proteins is probably due to the fusion protein being expressed at greater than physiological levels. This highlights the need to examine the role of these fusion proteins in a more relevant cell type such as primary haematopoietic progenitor cells.

In vivo models

Three different strategies have been employed to generate murine models of MLL leukaemias. These include the knock-in of the fusion partner into the endogenous *MLL* gene, over expression of the fusion gene by retroviral transduction of haematopoietic progenitor cells (HPCs) and the *de novo* creation of the translocation by engineering interchromosomal recombination between the *MLL* and partner gene loci.

The knock-in approach

The first model of MLL leukaemia was the development of an MLL-AF9 'knock-in' mouse (Corral *et al.*, 1996). Homologous recombination in ES cells was used to fuse the 3'-terminal human *AF9* sequence with exon 8 of *MLL* such that *MLL-AF9* expression was under the control of the endogenous murine *MLL* promoter. Chimeric mice were generated which developed AML between 4 and 9 months of age while mice with an *MLL-myc-tag* translocation (where the *myc-tag* was fused to exon 8 of *MLL*) failed to develop AML (Corral *et al.*, 1996). This finding suggests that truncation of MLL alone is not sufficient for leukaemogenesis and that the partner protein AF9 confers a gain of function upon MLL. A more detailed analysis over a longer observation period revealed that while the majority of the chimeric mice developed AML, a small percentage developed ALL (Dobson *et al.*, 1999). Although the MLL-AF9 translocation is predominantly associated with AML in humans, it is observed in a small percentage of ALL cases. Therefore, the MLL-AF9 knock-in model mimics the incidence of acute leukaemias found in patients with this translocation.

The retroviral transduction approach

The transduction of murine HPCs with retroviral MLL-fusion gene expression constructs is a more common method of generating murine models of MLL leukaemias. Haematopoietic progenitors enriched in HSCs were transduced with MLL-ENL and cultured in methylcellulose. The transduced cells were able to serially replate in methylcellulose and establish immortalised myeloid cell lines in liquid culture (Lavau *et al.*, 1997). However, HPCs transduced with a truncated MLL construct (containing only MLL sequence that is present in the translocation) or wild-type ENL, failed to replate beyond the second round (Lavau *et al.*, 1997). Importantly, the immortalised MLL-ENL cell lines induced AML (with a latency of 3-4 months) when transferred into syngeneic recipients (Lavau *et al.*, 1997). Subsequent studies have demonstrated that the fusion of MLL with ELL, AF10, AFX, FKHRL1 and CBP can immortalise HPCs and induce myeloid leukaemia when transferred into mice, although with differing latencies (Lavau *et al.*, 2000a; Lavau *et al.*, 2000b; DiMartino *et al.*, 2002; So and Cleary, 2002; So and Cleary, 2003). An overview of the retroviral transduction method is presented in Figure 1.5.

A model of acute biphenotypic leukaemia (ABL) which mimics the mixed-lineage phenotype of many of the human leukaemias associated with MLL-fusion proteins has been developed. Transduction of HPCs with MLL-ENL followed by culture in methylcellulose supplemented with cytokines that support B cell development yielded $B220^{+}$ $CD19^{-}$ immortalised cell lines (Zeisig *et al.*, 2003a). Transfer of these cell lines into syngeneic recipients resulted in the development of ABL within 3-4 months. The leukaemic cells possessed a $B220^{+}$ $CD19^{-}$ $Mac\text{-}1^{int}$ mixed lineage immunophenotype and were of a monocytoid morphology. In addition to their mixed-lineage immunophenotype, the cells co-expressed lymphoid specific and myeloid specific genes (Zeisig *et al.*, 2003a). Interestingly, MLL-GAS7 is the only MLL-fusion protein examined thus far that can induce AML, ALL or ABL in syngeneic recipients transplanted with freshly transduced HSCs (So *et al.*, 2003a).

The interchromosomal recombination approach

An interchromosomal recombination model has recently been developed which more accurately mimics the human MLL translocation than any of the aforementioned murine models. In this model the *de novo* translocation of *MLL* and

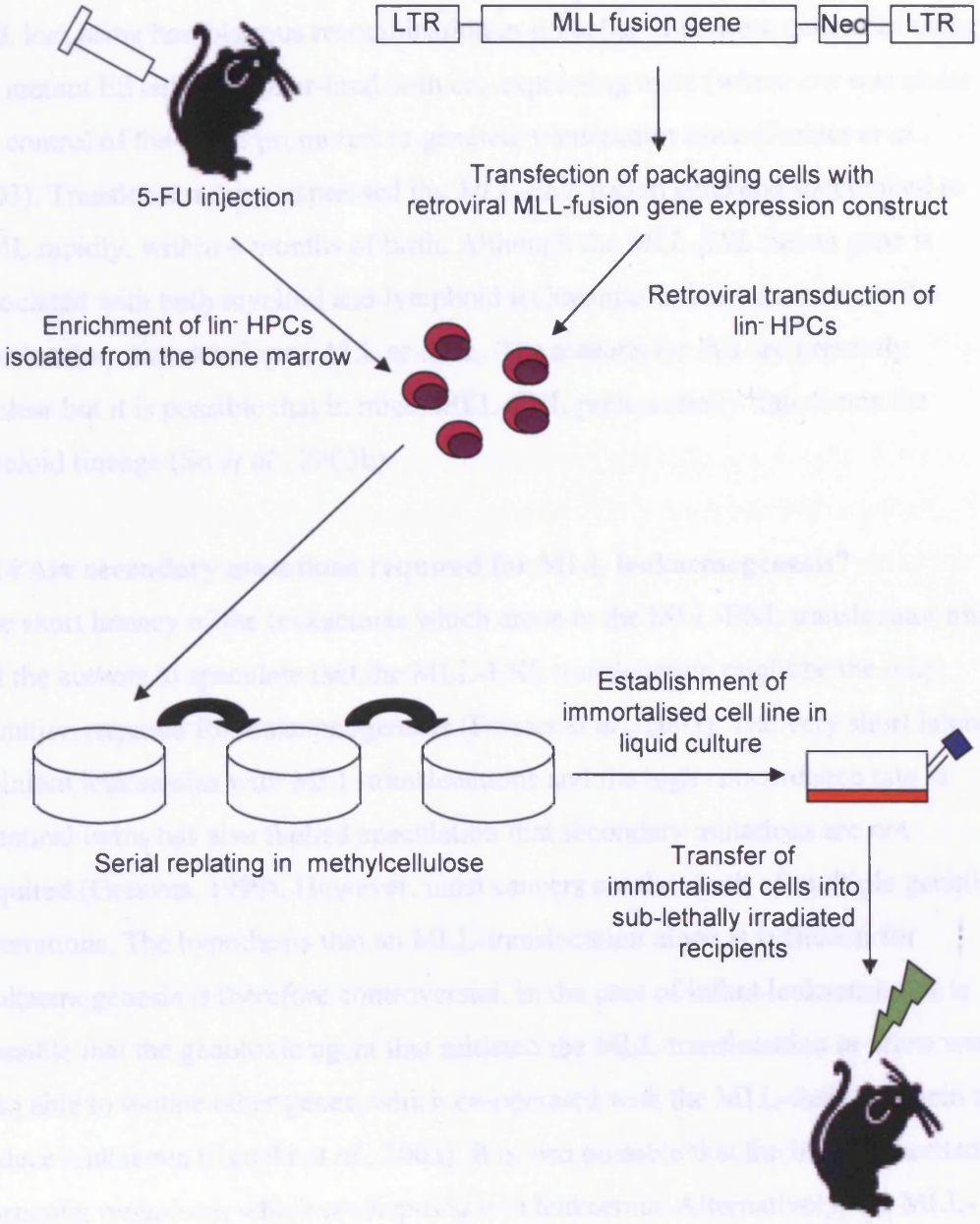

Figure 1.5 Experimental strategy of haematopoietic progenitor cell retroviral transduction. Lin$^-$ (lineage depleted) haematopoietic progenitor cells (HPCs) were isolated from the bone marrow of mice, 5 days after the injection of 5-fluorouracil (5-FU). Purified HPCs were transduced with an MLL-fusion gene retroviral expression construct and then serially replated in methylcellulose. Following the third replating, immortalised cells were maintained in liquid culture. The leukaemogenicity of the immortalised cells was measured by transfer into sub-lethally irradiated recipients. Adapted from Eguchi *et al.*, 2003.

ENL loci occurs specifically in haematopoietic cells and results in the generation of both reciprocal translocation products, which previous models have failed to recapitulate. This was achieved by engineering *loxP* sites into the murine *MLL* and *ENL* loci using homologous recombination in ES cells. Mice were generated using the mutant ES cells and inter-bred with cre-expressing mice (where *cre* was under the control of the *Lmo2* promoter) to generate translocator mice (Forster *et al.*, 2003). Translocator mice expressed the *MLL-ENL* fusion gene and succumbed to AML rapidly, within 4 months of birth. Although the *MLL-ENL* fusion gene is associated with both myeloid and lymphoid leukaemias in humans, none of the translocator mice developed ALL or ABL. The reasons for this are presently unclear but it is possible that in mice, MLL-ENL preferentially transforms the myeloid lineage (So *et al.*, 2003b).

1.14 Are secondary mutations required for MLL leukaemogenesis?

The short latency of the leukaemias which arose in the MLL-ENL translocator mice led the authors to speculate that the MLL-ENL translocation might be the only mutation required for leukaemogenesis (Forster *et al.*, 2003). The very short latency of infant leukaemias with MLL-translocations and the high concordance rate in identical twins has also fuelled speculation that secondary mutations are not required (Greaves, 1999). However, most cancers are the result of multiple genetic aberrations. The hypothesis that an MLL-translocation alone is sufficient for leukaemogenesis is therefore controversial. In the case of infant leukaemias, it is possible that the genotoxic agent that initiated the MLL-translocation *in utero* was also able to mutate other genes, which co-operated with the MLL-fusion protein to induce leukaemia (Eguchi *et al.*, 2003). It is also possible that the infant inherited particular mutations, which predisposed it to leukaemia. Alternatively, the MLL-fusion protein itself may directly influence the acquisition of secondary mutations (So and Cleary, 2002). Interestingly, it has recently been suggested that the de-regulated expression of the MLL target gene *Hoxa9* induces cytogenetic aberrations (Jordan and Guzman, 2004). Therefore, MLL-fusion proteins may promote genomic instability by de-regulating the expression of MLL target genes. Murine models of MLL leukaemias have demonstrated that particular MLL-fusion proteins induce leukaemia with substantially different latencies. For example, MLL-ENL, MLL-AF9, MLL-GAS7, MLL-AF1p and MLL-AF10 transduced cells required

between 3 and 5 months to give rise to leukaemia in syngeneic recipients (DiMartino *et al.*, 2002; Cozzio *et al.*, 2003; Martin *et al.*, 2003; So *et al.*, 2003a; So *et al.*, 2003b). In contrast, MLL-ELL, MLL-AFX, MLL-FKHRL1 and MLL-CBP transduced cells gave rise to leukaemia after a much longer latency (Lavau *et al.*, 2000a; Lavau *et al.*, 2000b; So and Cleary, 2003). These studies suggest that MLL-fusion proteins differ in their oncogenecity or their requirement for secondary mutations.

If secondary mutations are required for MLL leukaemias, it is possible that the leukaemic cells would no longer be dependent on the MLL-fusion protein for their survival. Conditional systems of MLL-fusion protein expression have been developed in order to investigate whether immortalised cells are dependent on continued expression of the MLL-fusion protein. HPCs immortalised using an oestrogen regulated conditional MLL-ENL fusion protein were dependent on the continued presence of tamoxifen for their survival and proliferation (Zeisig *et al.*, 2004). The withdrawal of tamoxifen and subsequent inactivation of MLL-ENL resulted in the terminal differentiation of the immortalised cells into neutrophils. Therefore, MLL-ENL is required to maintain the immortalised phenotype *in vitro*. However, it remains to be determined whether MLL-ENL is required to maintain leukaemia *in vivo*.

Several studies have examined the effect of targeting the expression of the *MLL*-fusion genes in cell lines derived from patient material. The targeted down-regulation of the MLL-AF9 transcript in the THP-1 cell line was achieved using antisense oligonucleotides and resulted in reduced *HOXA7* and *HOXA10* expression, reduced proliferation and some apoptosis (Kawagoe *et al.*, 2001). Similar results were obtained following the down-regulation of MLL-ENL transcript expression in the KOCL33 cell line (Akao *et al.*, 1998). The inhibition of THP-1 cell growth was not accompanied by terminal differentiation and the down-regulation of MLL-AF9 did not restore the ability of the cells to differentiate in response to G-CSF or M-CSF (Pession *et al.*, 2003). Hence, although MLL-AF9 promotes the proliferation of leukaemic cells and protects them from apoptosis, it does not block their differentiation. It is likely that mutations other than MLL-AF9

contribute to a block in differentiation of these leukaemic cells (Pession *et al.*, 2003).

1.15 The mechanism of action of MLL fusion proteins

The models of MLL leukaemias described above have provided important insights into the mechanism of action of MLL fusion proteins. Knock-in mice which were engineered to express an *MLL-myc tag* only possess one functional copy of *MLL* yet they do not develop leukaemia (Corral *et al.*, 1996; Dobson *et al.*, 1999). Therefore, it is unlikely that *MLL* haploinsufficiency promotes leukaemogenesis. Furthermore, MLL-fusion proteins can immortalise HPCs in the presence of two copies of *MLL* (Lavau *et al.*, 1997). The simple truncation of MLL or the over-expression of ENL is not sufficient to immortalise HPCs suggesting that the partner protein confers a gain of function to MLL (Lavau *et al.*, 1997).

A possible dominant negative effect of MLL-fusion proteins on normal MLL function has been inferred from the finding that the over-expression of an N-terminal truncated MLL protein, but not full-length MLL, can block G-CSF induced differentiation of 32Dc13 cells (Joh *et al.*, 1996). However, *MLL-AF9* knock-in mice, which possess one translocated *MLL* allele and one wild-type *MLL* allele, are viable (Dobson *et al.*, 1999). Since the *MLL* null mutation is embryonic lethal (Yu *et al.*, 1995; Yagi *et al.*, 1998), the production of viable *MLL-AF9* heterozygote mice suggests that MLL fusion proteins do not act as dominant negative inhibitors of normal MLL function (Ayton and Cleary, 2001). However, there is evidence to suggest that MLL fusion proteins can act as dominant negative inhibitors of partner protein function. For example, fusion of MLL to the forkhead transcription factor AFX suppressed the ability of the wild-type forkhead protein FKHRL1 to induce the apoptosis of Ba/F3 cells (So and Cleary, 2002). Furthermore, the C-terminus of AFX was able to enhance the self-renewal of HPCs, although not to the same extent as MLL-AFX. The fact that the MLL-AFX fusion protein and the C-terminus of AFX lack an intact forkhead DNA binding domain suggests that they may compete with wild-type forkhead proteins for essential cofactors (So and Cleary, 2002). Therefore, MLL-AFX and MLL- FKHRL1 may disrupt the activity of the AFX and FKHRL1 forkhead proteins, which normally function to suppress cell growth. The MLL partner proteins GAS7, Abi-1, AF6 and

AF1p also function to suppress cell growth, suggesting that fusion with MLL may disrupt their activity and promote leukaemogenesis (So and Cleary, 2002; So *et al.*, 2003b).

Taken together, the knock-in model and the retroviral transduction model have demonstrated that the MLL fusion partner confers critical effector domains to MLL, which are required for leukaemogenesis. These studies therefore support a gain of function model as the mechanism of action of MLL fusion proteins. In addition, the MLL-fusion protein may act as a dominant negative inhibitor of the normal function of the partner protein in some cases, which may also contribute to leukaemogenesis.

1.16 The transcriptional transactivation domains of nuclear MLL fusion partners are essential for leukaemogenesis

Deletion mutagenesis studies of various *MLL*-fusion genes have been performed in order to gain insight into the mechanism of HPC immortalisation. Deletion of various sections of *MLL-ENL* revealed that the AT hooks and the MT domain of MLL and the transcriptional transactivation domain of ENL were essential for immortalisation (Slany *et al.*, 1998). A more comprehensive study revealed that the AT hooks were dispensable but the MT domain, as well as a novel domain with unknown function in the N-terminus and an uncharacterised central region of MLL, were required for immortalisation by MLL-ENL (Ayton *et al.*, 2004). Interestingly, the ability of MLL-ENL to immortalise HPCs was strictly dependent on an intact CXXC domain, which mediates the binding of MLL to unmethylated CpG sequences (Ayton *et al.*, 2004).

The region of ENL encompassing the transcriptional transactivation domain encodes two helical structures, which are highly conserved in AF9. The ability of the C-terminus of ENL to act as a transcriptional activator was confirmed in reporter gene assays. The C-terminus of MLL-ENL and the MT domain of MLL were required in order for MLL-ENL to transactivate the minimal *SV40* and *Hoxa7* promoters (Schreiner *et al.*, 1999). Therefore, although the MT domain of MLL is a transcriptional repressor (Zeleznik-Le *et al.*, 1994), fusion with ENL results in the generation of a potent transcriptional activator (Schreiner *et al.*, 1999).

The fusion of MLL with a variety of transactivator and repressor domains, confirmed that transactivator properties donated by the fusion partner are required for the oncogenic effects of some MLL-fusion proteins. Interestingly, a perfect correlation between the transactivation capacity and the transformation ability of each fusion gene was observed. The MLL-VP16 fusion protein, in which MLL was fused to the complete Herpes simplex VP16 activation domain, transactivated the minimal *SV40* and *Hoxa7* promoters and immortalised HPCs (Zeisig *et al.*, 2003b). However, MLL-VP16 did not transactivate these promoters as well as the authentic MLL-ENL fusion protein and the MLL-VP16 immortalised cells grew more slowly and exhibited a phenotype which was more mature than that of MLL-ENL immortalised cells. Fusion of MLL with the GAL4 activation domain, the acidic transactivator sequence AD42 or the KRAB repressor domain failed to transactivate the *SV40* and *Hoxa7* promoters as well as MLL-VP16 and failed to immortalise HPCs (Zeisig *et al.*, 2003b). This data and the fact that most, if not all, nuclear fusion partners of MLL possess transactivation domains or domains that enable their interaction with transcriptional activators, suggests that the fusion partner confers novel transcriptional regulatory activity on MLL, resulting in de-regulation of wild-type MLL target genes and thus leukaemogenesis.

1.17 The dimerisation domains of cytoplasmic MLL fusion partners are essential for leukaemogenesis.

The observation that many of the MLL fusion partners are cytoplasmic with no known roles in transcription, suggests that these partner proteins confer a different activity to MLL. Some of the cytoplasmic MLL fusion partners such as AF1p and GAS7 contain coiled-coil dimerisation motifs (Cupers *et al.*, 1997; She *et al.*, 2002). A current hypothesis is that these dimerisation motifs contribute to the leukaemogenic activity of the MLL fusion protein. This hypothesis was based on the finding that a significant number of mice engineered to express an artificial *MLL-β-galactosidase* (*LacZ*) fusion gene developed leukaemia, yet mice expressing an *MLL-myc-tag* did not succumb to the disease (Dobson *et al.*, 2000). While the phenotype of the leukaemia was similar to that of the *MLL-AF9* knock-in mice (Corral *et al.*, 1996), the latency and penetrance of the disease was very different. Whereas the majority of the *MLL-AF9* chimeric mice had succumbed to the disease by 12 months, only 35% of the *MLL-LacZ* chimeric mice had developed leukaemia

by 20 months (Corral *et al.*, 1996; Dobson *et al.*, 2000). The β-galactosidase enzyme normally functions as a tetramer which suggests that the MLL-β-galactosidase fusion protein may also exist as a tetramer (Dobson *et al.*, 2000). Hence, β-galactosidase may confer dimerisation motifs to MLL which alters its activity and results in leukaemogenesis.

The dimerisation hypothesis is also supported by several other studies. A conditional MLL construct, which could be induced to dimerise by a pharmacological agent, was created by fusing N-terminal truncated MLL with the FK506 binding protein (FKBP). The ability of this construct to activate transcription and immortalise HPCs was strictly dependent on the presence of the dimerising agent (Martin *et al.*, 2003). Internal N-terminal partial tandem duplications of *MLL* are found in a subset of patients with AML (Caligiuri *et al.*, 1998). Interestingly, an MLL construct in which the N-terminal sequences spanning the AT hook and MT domains were duplicated, activated reporter gene expression to the same extent as dimerised MLL-FKBP (Martin *et al.*, 2003). Therefore, exon duplicated MLL and dimerised MLL may function in a similar way. The dimerised form of MLL and exon duplicated MLL should have twice as many DNA binding motifs as the undimerised form. For example, the dimerised protein will have two AT hook domains and two MT domains. Dimerised MLL may therefore possess a greater affinity for target gene promoters or accessory co-factors than wild-type MLL, which may explain its enhanced transcriptional activity. The observation that MLL-FKBP binding to the *Hoxa9* promoter was increased in the presence of dimeriser is in agreement with this hypothesis (Martin *et al.*, 2003).

The coiled-coil domains of the MLL fusion partners AF1p, GAS7 and SEPTIN6 facilitate their homo-dimerisation and are essential for MLL-AF1p, MLL-GAS7 and MLL-SEPT6 mediated transformation (So *et al.*, 2003b; Ono *et al.*, 2005). Although truncated MLL, AF1p and GAS7 possessed little transcriptional transactivation ability themselves, the MLL-AF1p and MLL-GAS7 fusion proteins were potent transcriptional activators, capable of transactivating the *Hoxa7* promoter over 50-fold (So *et al.*, 2003b). Hence, the role of both the nuclear and cytoplasmic MLL fusion partners may be to convert MLL into a constitutive transcriptional activator (Figure 1.6). In agreement with this hypothesis, MLL-

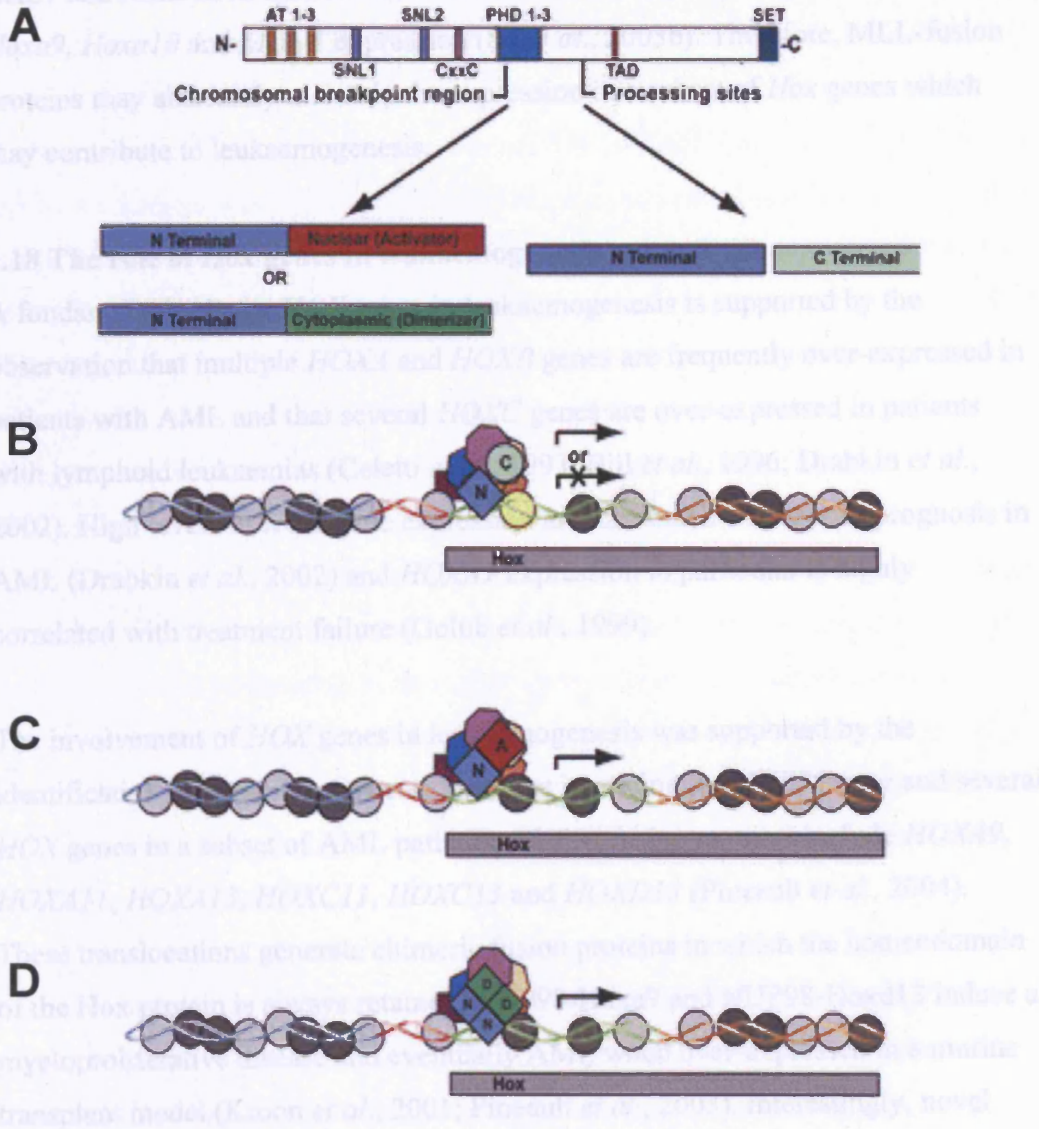

Figure 1.6 Mechanisms of *Hox* gene regulation by MLL fusion proteins. A) Wild-type MLL is cleaved at the proteolytic cleavage site by Taspase 1 into N- and C-terminal peptides. Following chromosomal translocation, the N-terminus of MLL is fused to the C-terminus of either nuclear or cytoplasmic fusion partners. AT1-3: AT hooks, SNL1 and 2: sub-nuclear localisation domains, CxxC: cysteine rich methyltransferase homology domain, PHD1-3: PHD domain. **B)** Gene regulation by wild-type MLL. The processed N- and C-terminal peptides of MLL interact in a multi-protein super-complex which remodels the chromatin of *Hox* genes such that their expression is maintained in haematopoietic progenitors (grey circles: unmethylated histones, yellow circles: methylated histones). During haematopoietic differentiation, polycomb group proteins may antagonise the function of MLL such that *Hox* gene expression is no longer maintained. **C)** MLL-fusion proteins may act as monomers to constitutively activate *Hox* gene expression if the fusion-partner possesses transactivation properties. **D)** Alternatively, MLL-fusion proteins may act as dimers to constitutively activate *Hox* gene expression if the fusion partner possesses dimerisation motifs. Reproduced from Hsu and Look, 2003.

GAS7 and MLL-ENL immortalised cells exhibited very similar patterns of *Hoxa7*, *Hoxa9*, *Hoxa10* and *Meis-1* expression (So *et al.*, 2003b). Therefore, MLL-fusion proteins may aberrantly maintain the expression of a subset of *Hox* genes which may contribute to leukaemogenesis.

1.18 The role of *Hox* genes in leukaemogenesis

A fundamental role for *HOX* genes in leukaemogenesis is supported by the observation that multiple *HOXA* and *HOXB* genes are frequently over-expressed in patients with AML and that several *HOXC* genes are over-expressed in patients with lymphoid leukaemias (Celetti *et al.*, 1993; Bijl *et al.*, 1996; Drabkin *et al.*, 2002). High levels of *HOX* gene expression are associated with a poor prognosis in AML (Drabkin *et al.*, 2002) and *HOXA9* expression in particular is highly correlated with treatment failure (Golub *et al.*, 1999).

The involvement of *HOX* genes in leukaemogenesis was supported by the identification of chromosomal translocations involving the *NUP98* gene and several *HOX* genes in a subset of AML patients. *NUP98* fusion partners include *HOXA9*, *HOXA11*, *HOXA13*, *HOXC11*, *HOXC13* and *HOXD13* (Pineault *et al.*, 2004). These translocations generate chimeric fusion proteins in which the homeodomain of the Hox protein is always retained. NUP98-Hoxa9 and NUP98-Hoxd13 induce a myeloproliferative disease and eventually AML when over-expressed in a murine transplant model (Kroon *et al.*, 2001; Pineault *et al.*, 2003). Interestingly, novel NUP98-Hox fusion proteins, which are not found in patients such as NUP98-Hoxa10 and NUP98-Hoxb3, are also leukaemogenic in mouse models (Pineault *et al.*, 2004). However, the NUP98-Hoxb4 fusion protein is not. This finding is consistent with previous studies in which Hoxa10 and Hoxb3 but not Hoxb4 are leukaemogenic when over-expressed in a murine transplant model (Sauvageau *et al.*, 1995; Sauvageau *et al.*, 1997; Thorsteinsdottir *et al.*, 1997). Collectively these data suggest that many, but not all, *Hox* genes are potentially leukaemogenic.

NUP98-Hox fusion proteins induce AML after a long latency which is usually monoclonal and preceded by a myeloproliferative disorder. This suggests that secondary mutations are required for disease progression. Interestingly, the latency of AML onset is dramatically reduced by the co-expression of *Meis-1* (Pineault *et*

al., 2004). Meis-1 (myeloid ecotropic viral insertion site 1) is a non-Hox homeodomain containing transcription factor that acts as a co-factor for Hox proteins. It was originally identified as a common site of retroviral integration in BXH-2 myeloid leukaemias (Moskow *et al.*, 1995). BXH-2 mice carry an ecotropic murine leukaemia virus that acts as an insertional mutagen. Therefore, these mice can be used to identify genes which co-operate to induce leukaemia (Moskow *et al.*, 1995). Retroviral integrations at the *Meis-1* locus occurred in approximately 15% of BXH-2 leukaemias, of which 95% also contained activating retroviral integrations in the *Hoxa7* or *Hoxa9* genes (Nakamura *et al.*, 1996a). This finding implied that Meis-1 co-operates with Hoxa9 or Hoxa7 to induce leukaemia. Indeed, Meis-1 is a common collaborator in leukaemogenesis. Although, *Meis-1* over-expression alone is not sufficient for leukaemogenesis, the over-expression of *Meis-1* in concert with *Hoxa9*, *Hoxa10*, *Hoxb3*, *Hoxb4* or *Hoxb6* induces AML with a much shorter latency than over-expression of these *Hox* genes alone (Thorsteinsdottir *et al.*, 1997; Kroon *et al.*, 1998; Thorsteinsdottir *et al.*, 2001; Pineault *et al.*, 2004; Fischbach *et al.*, 2005). Meis-1 is required for normal haematopoietic development and is thought to play a role in regulating the proliferation or self-renewal of the HSC (Hisa *et al.*, 2004).

Since *Hox* gene over-expression blocks the differentiation of haematopoietic progenitor cells (Calvo *et al.*, 2000; Pineault *et al.*, 2004), a current hypothesis is that Meis-1 co-operates with this differentiation block by increasing the proliferative or self-renewal capacity of these immortalised cells (Pineault *et al.*, 2005). Consistent with this hypothesis, a recent study found that the over-expression of *Meis-1* in Hoxa9 immortalised progenitor cells activated the expression of HSC specific genes such as *Flt-3* and *CD34* (Wang *et al.*, 2005). This finding implies that the over-expression of Meis-1 can convert immortalised progenitor cells into leukaemic stem cells. Another study found that the over-expression of *Hoxa9* and *Meis-1* protected several myeloid and lymphoid cell lines from pro-apoptotic stimuli (Wermuth and Buchberg, 2005). Therefore, Hoxa9 and Meis-1 may cooperate to confer a protection from apoptosis which may provide a strong selective advantage to cells and ultimately promote leukaemogenesis.

1.19 Identification of MLL-fusion protein target genes

Much emphasis has been placed on the role of *Hox* genes in MLL leukaemias. This is because MLL-fusion proteins are thought to de-regulate the expression of wild-type MLL target genes and the only known targets of MLL to date are a subset of *Hox* genes.

Gene expression profiling of patient samples

Gene expression profiling of patient material has revealed that leukaemias with MLL translocations possess a unique gene expression profile which can distinguish them from other myeloid or lymphoid leukaemias lacking MLL translocations (Armstrong *et al.*, 2002). High levels of *HOXA4*, *HOXA5*, *HOXA9*, *HOXA10*, *HOXC6*, *MEIS-1* and *PBX-3* expression were observed in *MLL* rearranged B-precursor ALL but not in B-precursor ALL lacking *MLL* rearrangement (Armstrong *et al.*, 2002; Andersson *et al.*, 2005). In addition, *HOXA9*, *HOXA10*, *HOXC6* and *MEIS-1* were differentially expressed in T-lineage ALL with *MLL* rearrangements (Ferrando *et al.*, 2003). High levels of *HOXA4*, *HOXA5*, *HOXA9*, *HOXA10*, *MEIS-1* and *PBX-3* have also been reported in *MLL* rearranged myeloid leukaemias (Debernardi *et al.*, 2003; Ross *et al.*, 2004). Therefore, a popular hypothesis is that MLL-fusion proteins establish a *HOX* transcriptional programme, which contributes to leukaemogenesis (Armstrong *et al.*, 2003). However, *HOXA9* and *MEIS-1* are frequently co-expressed in AML and are not specific to AML with *MLL* rearrangements. Instead co-activation of *HOXA9* and *MEIS-1* is a common event in AML (Lawrence *et al.*, 1999).

Inducible systems of *MLL*-fusion gene expression

Although common gene expression signatures can be identified by analysing patient samples, it is not possible to discriminate between genes which are targets of the MLL-fusion protein and those which are up-regulated as the leukaemic cells acquire subsequent mutations. Conditional systems of MLL-fusion protein expression are more useful in this respect. The gene expression profile of HPCs immortalised by an oestrogen-regulated conditional MLL-ENL fusion protein was examined by microarray analysis following treatment with or without tamoxifen (Zeisig *et al.*, 2004). Some of the genes down-regulated after the withdrawal of tamoxifen (when MLL-ENL was inactivated) were over-expressed in patients with

MLL-rearrangements. These included *Flt-3*, *Meis-1*, *Hoxa9* and *Lmo2*. Further analysis using real-time PCR revealed that *Hoxa7* was also expressed in the immortalised cell lines and down-regulated when MLL-ENL was inactivated (Zeisig *et al.*, 2004). The authors concluded that the genes down-regulated upon inactivation of MLL-ENL were targets of MLL-ENL. However, an alternative explanation is that the over-expression of *Hoxa9* and *Meis-1* in MLL-ENL immortalised cell lines is a function of the stage of differentiation of the immortalised cell. Hence, it is possible that the expression of these genes decreased upon the withdrawal of tamoxifen because the immortalised cells differentiated upon loss of MLL-ENL expression. Therefore, *Hoxa9* and *Meis-1* may not necessarily be direct targets of MLL-ENL. Similar experiments using HPCs immortalised by a conditional MLL dimerisation construct revealed that expression of *Hoxa7*, *Hoxa9* and *Meis-1* decreased following the withdrawal of dimeriser. However, the re-addition of dimeriser resulted in the up-regulation of these genes, which suggests that these genes are targets of dimerised MLL (Martin *et al.*, 2003).

In order to determine if *Hoxa9* and *Meis-1* were targets of MLL-ENL, these genes were over-expressed in MLL-ENL immortalised cells and then MLL-ENL was inactivated by the withdrawal of tamoxifen (Zeisig *et al.*, 2004). The authors proposed that if these genes were targets of MLL-ENL then they should be able to substitute for MLL-ENL expression when MLL-ENL was inactivated. The over-expression of *Hoxa9*, *Hoxa7* or *Meis-1* alone was not able to prevent the terminal differentiation observed when MLL-ENL was inactivated. In contrast, the co-expression of *Hoxa9* and *Meis-1* was sufficient to maintain the immortalised phenotype when MLL-ENL was inactivated (Zeisig *et al.*, 2004). Therefore, the authors concluded that *Hoxa9* and *Meis-1* were targets of MLL-ENL. However, they failed to account for the fact that the immortalised cell line 'rescued' by the over-expression of *Hoxa9* and *Meis-1*, possessed a more mature phenotype than that of the parental cell line originally immortalised by MLL-ENL. Previous studies have shown that the co-expression of *Hoxa9* and *Meis-1* can immortalise HPCs *in vitro* and *in vivo* (Calvo *et al.*, 2001; Thorsteinsdottir *et al.*, 2001). Therefore, it is possible that alleviation of the differentiation block imposed by MLL-ENL by the withdrawal of tamoxifen, allowed the cells to differentiate up to a point at which they were susceptible to the differentiation block imposed by Hoxa9 and Meis-1

and effectively re-immortalised. In this case, over-expression of *Hoxa9* and *Meis-1* would not necessarily be substituting for MLL-ENL expression. Similar studies have shown that the over-expression of *AML1-ETO*, *PML-RARα*, *NUP98-Hoxa9*, *Hoxa9*, *Hoxb8* or *Hoxa7* prevented the terminal differentiation of conditional E2A-PBX1 myeloid immortalised cell lines upon the withdrawal of tamoxifen (Sykes and Kamps, 2001). Importantly, the differentiation block imposed by each oncogene was unique and different to that originally imposed by E2A-PBX1 (Sykes and Kamps, 2001), which suggests that these oncogenes were not substituting for E2A-PBX1 expression.

Aims

The aim of my project was to establish the molecular mechanism by which MLL-ENL induces leukaemia. I used retroviral delivery in combination with the Tet-Off conditional expression system. I immortalised murine HPCs with MLL-ENL and then turned off expression of the fusion protein by the addition of doxycycline (dox). This system allowed me to determine whether continued expression of MLL-ENL was required to maintain immortalisation *in vitro*. It also allowed me to investigate whether continued expression of MLL-ENL was required to maintain the leukaemia *in vivo*. This was achieved by transferring the immortalised cells into mice and administering dox once the mice displayed signs of leukaemia. Finally, this system enabled me to identify targets of MLL-ENL and thus establish its oncogenic mechanism.

Chapter 2 Materials and Methods

Table 2.1 Buffers used in this study.

Buffer	Components
Erythrocyte lysis buffer	17 mM Tris (pH 7.2); 0.144 M NH_4Cl
Stain buffer (flow cytometry)	PBS; 0.5% BSA; 0.05% sodium azide
RIPA lysis buffer	150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 50 mM Tris (pH 8.0); 5 mM EDTA
NP40 lysis buffer	150mM NaCl; 0.5% NP40; 50mM Tris (pH 8.0)
5 x protein sample buffer	500 mM DTT; 10% SDS; 312.5 mM Tris (pH 6.8); 0.05% Bromophenol Blue; 25% Glycerol
1 x running buffer	0.192 M glycine; 25 mM Tris; 0.1% SDS
1 x transfer buffer	9.5 mM CAPS (pH 11.0)
1 x stripping buffer	62.5 mM Tris (pH 6.8); 1% SDS; 50 mM 2-mercaptoethanol
TNES	TE (pH 8.0); 0.1 mM NaCl; 1% SDS
Denaturing solution	1.5 M NaCl; 0.5 M NaOH
Neutralising solution	1 M Tris (pH 7.4); 1.5 M NaCl
5 x probe labelling buffer	0.5 M Na_2HPO_4; 0.5 M NaH_2PO_4; 0.5% 2-ME; 1 mM of dATP, dGTP and dTTP
Pre-hybridisation solution	5 x SSC; 0.5% SDS; 0.1 g/ml dextran sulphate; 5 x Denhardt's solution
TE (pH 8.0)	10 mM Tris (pH 8.0); 1 mM EDTA (pH 8.0)

2.1 Retroviral constructs

Diagrams of all the constructs described in this section are shown in Figures 2.1 and 2.2. All of the MLL-fusions were sequenced prior to sub-cloning by Dr. Dale Moulding and Dr. Inusha de Silva of the Molecular Haematology and Cancer Biology (MHCB) unit. Comprehensive restriction digests of the constructs were performed and DNA sequencing around the cloning sites of the MLL-fusion constructs confirmed that errors had not been introduced during the sub-cloning process.

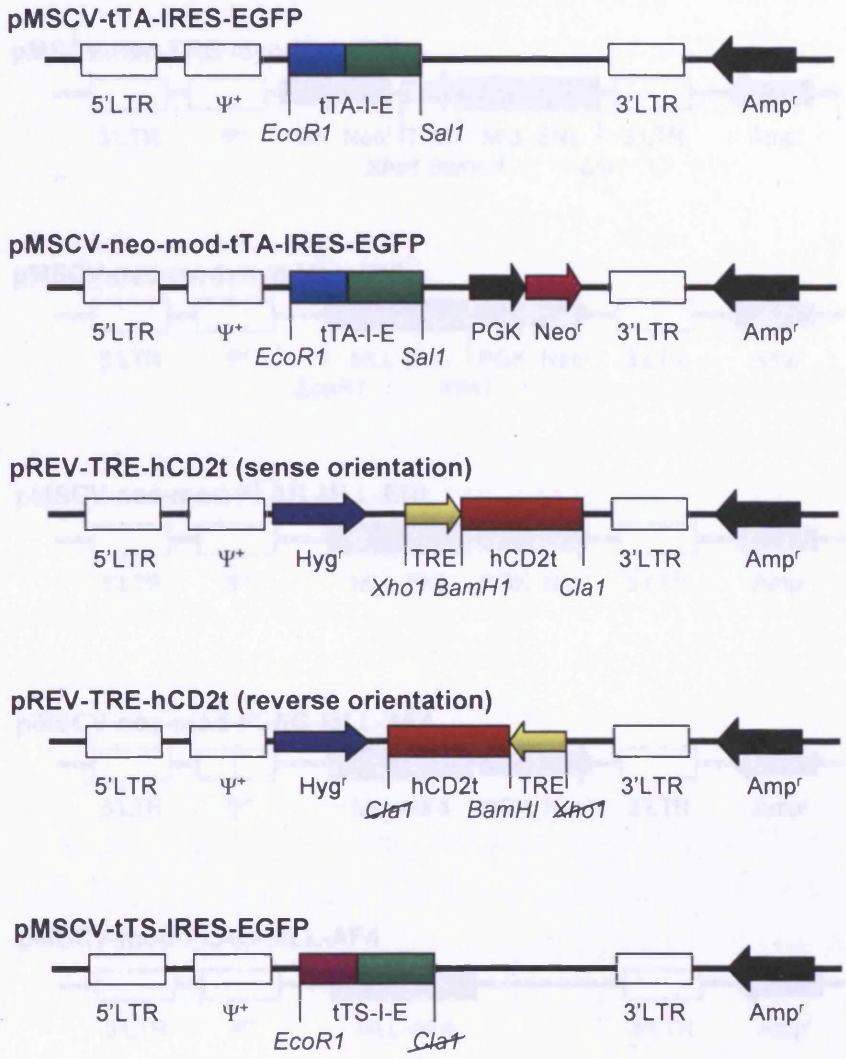

Figure 2.1 A diagram of the retroviral reporter constructs used in this study. Restriction sites that are crossed out indicate that the site was destroyed by filling in the over-hang with Klenow and by performing a blunt-end ligation. tTA-I-E: tTA- (tetracycline transactivator) IRES- (internal ribosome entry site), EGFP- (enhanced green fluorescent protein), tTS-I-E: tTS- (tetracycline suppressor)-IRES-EGFP, hCD2t: human CD2 tail-less gene, TRE: tetracycline response element, LTR: long terminal repeat, Ψ^+: viral packaging signal, PGK: phosphoglycerate kinase promoter, Neo^r: neomycin resistance gene, Hyg^r: hygromycin resistance gene, Amp^r: ampicillin resistance gene.

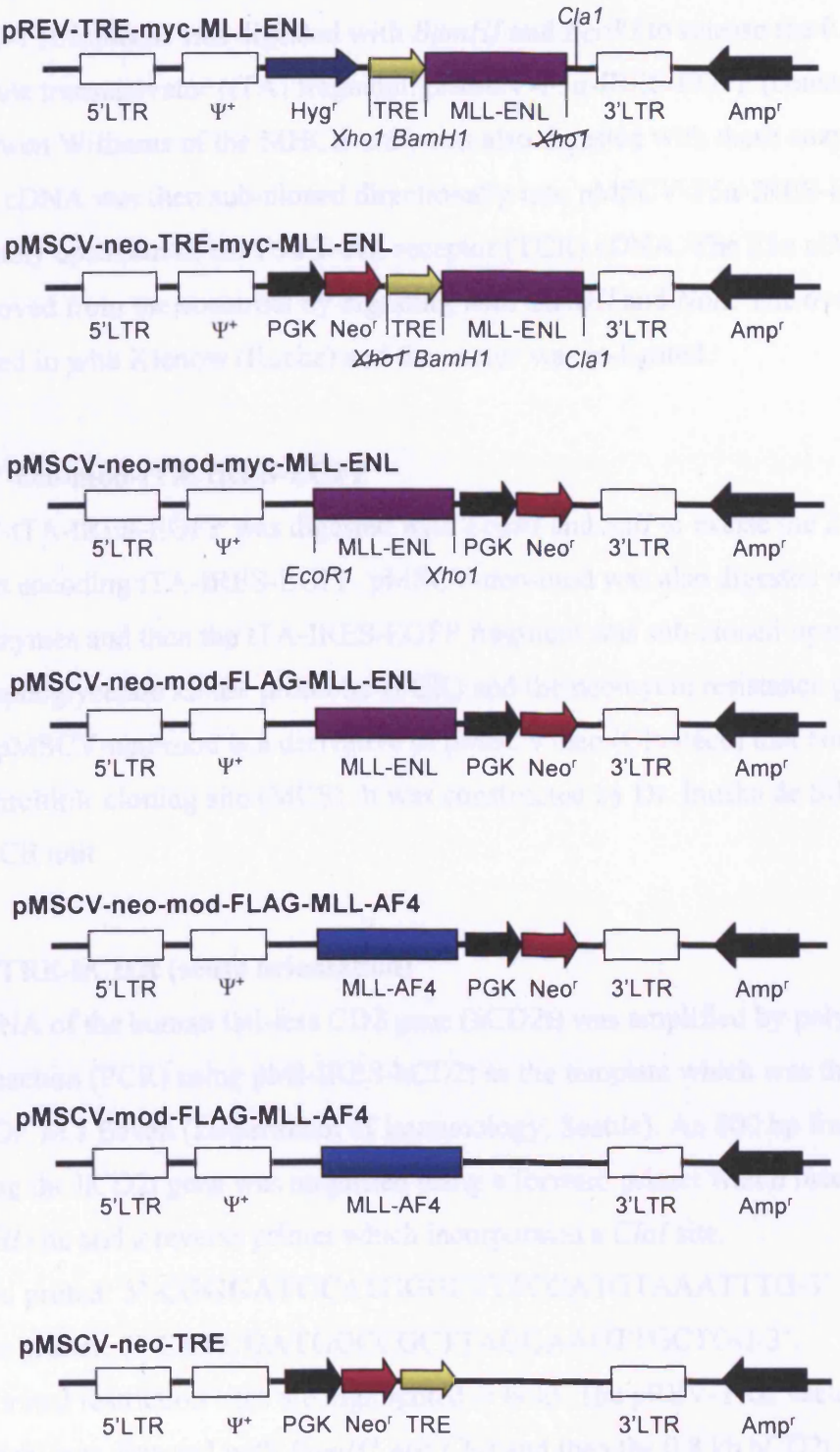

Figure 2.2 A diagram of the MLL-fusion gene retroviral constructs used in this study. Restriction sites that are crossed out indicate that the site was destroyed by filling in the over-hang with Klenow and by performing a blunt-end ligation. TRE: tetracycline response element, LTR: long terminal repeat, Ψ^+: viral packaging signal, PGK: phosphoglycerate kinase promoter, Neo^r: neomycin resistance gene, Hyg^r: hygromycin resistance gene, Amp^r: ampicillin resistance gene.

pMSCV-tTA-IRES-EGFP

pUHT 61-1 (Clontech) was digested with *BamHI* and *EcoRI* to release the 0.76 kb tetracycline transactivator (tTA) fragment. pMSCV-F5α-IRES-EGFP (constructed by Dr. Owen Williams of the MHCB unit) was also digested with these enzymes. The tTA cDNA was then sub-cloned directionally into pMSCV-F5α-IRES-EGFP, immediately upstream of the F5α T-cell receptor (TCR) cDNA. The F5α cDNA was removed from the construct by digesting with *BamHI* and *NotI*. The over-hangs were filled in with Klenow (Roche) and the vector was re-ligated.

pMSCV-neo-mod-tTA-IRES-EGFP

pMSCV-tTA-IRES-EGFP was digested with *EcoRI* and *SalI* to excise the 2.1 kb fragment encoding tTA-IRES-EGFP. pMSCV-neo-mod was also digested with these enzymes and then the tTA-IRES-EGFP fragment was sub-cloned upstream of the phosphoglycerate kinase promoter (PGK) and the neomycin resistance gene (Neo^r). pMSCV-neo-mod is a derivative of pMSCV-neo (Clontech) that contains an altered multiple cloning site (MCS). It was constructed by Dr. Inusha de Silva of the MHCB unit.

pREV-TRE-hCD2t (sense orientation)

The cDNA of the human tail-less CD2 gene (hCD2t) was amplified by polymerase chain reaction (PCR) using pMI-IRES-hCD2t as the template which was the kind gift of Dr. M.J Bevan (Department of Immunology, Seattle). An 800 bp fragment encoding the hCD2t gene was amplified using a forward primer which incorporated a *BamHI* site and a reverse primer which incorporated a *ClaI* site.
Forward primer: 5'-CG**GGATCC**ATGGGCTTTCCATGTAAATTTG-3'
Reverse primer: 5'-C**ATCGAT**GGCCGCTTAGGAAGTTGCTGG-3'.
Incorporated restriction sites are highlighted in bold. The pREV-TRE vector (Clontech) was digested with *BamH1* and *ClaI* and then the 0.8 kb hCD2t PCR product was sub-cloned immediately downstream of the tetracycline response element (TRE).

pREV-TRE-hCD2t (reverse orientation)

pREV-TRE-hCD2t (sense) was digested with *ClaI*. The *ClaI* over-hangs were filled in with Klenow and then the vector was digested with *XhoI* to release the 1.2 kb fragment encoding TRE-hCD2t. Meanwhile, the pREV-TRE vector was digested with *XhoI*, filled in with Klenow and then digested with *SalI*. This digestion removed TRE and allowed the directional sub-cloning of the 1.2 kb TRE-hCD2t fragment downstream of the hygromycin resistance gene (hyg^r).

pMSCV-tTS-IRES-EGFP

pTet-tTS (Clontech) was digested with *ClaI*. The over-hangs were filled in with Klenow and the 0.86 kb tetracycline suppressor (tTS) fragment was excised by digestion with *EcoRI*. Meanwhile, the pMSCV-F5α-IRES-EGFP construct was digested with *NotI*, filled in with Klenow and then digested with *EcoRI*. This digestion removed the F5α cDNA and allowed the directional sub-cloning of tTS upstream of the internal ribosome entry site (IRES) and the gene encoding enhanced green fluorescent protein (EGFP).

pREV-TRE-myc-MLL-ENL

The pCSARQ2 construct containing the MLL-ENL cDNA (aa 27-1444 of MLL and aa 5-559 of ENL) was a gift from D. C. Tkachuk (Princess Margaret Hospital, Toronto, Canada (Adler et al., 1997)]. This construct contains 6 myc tags at the 5' end of MLL-ENL and 1 HA tag at the 3' end. In order to sub-clone MLL-ENL into pREV-TRE, pCSARQ2 was digested with *ClaI* and the overhangs were filled in with Klenow. The 6.24 kb fragment encoding MLL-ENL was then excised by digestion with *XhoI*. Meanwhile, pREV-TRE was digested with *BamHI*, filled in with Klenow and then digested with *SalI*. MLL-ENL was then sub-cloned directionally into pREV-TRE, immediately downstream of the TRE. The *BamHI* site still exists at the start of MLL-ENL since ligation of a blunted *BamHI* to a blunted *ClaI* site recreated the *BamHI* site.

pMSCV-neo-TRE-myc-MLL-ENL

pREV-TRE-myc-MLL-ENL was digested with *XhoI* and *ClaI* to release the 6.7 kb TRE-MLL-ENL fragment. pMSCV-neo was digested with *SalI* and *ClaI* and then the TRE-MLL-ENL fragment was sub-cloned downstream of the PGK-Neo^r.

pMSCV-neo-mod-myc-MLL-ENL

pCSARQ2 was digested with *ClaI* and the over-hangs were filled in with Klenow. The 6.24 kb MLL-ENL cDNA fragment was then excised by digestion with *XhoI*. pMSCV-neo-mod was digested with *EcoRI*, filled in with Klenow and then digested with *XhoI*. The MLL-ENL cDNA was then sub-cloned into pMSCV-neo-mod upstream of the PGK-Neo^r. The *EcoRI* site still exists at the start of MLL-ENL since ligation of a blunted *EcoRI* site to a blunted *ClaI* site recreated the *EcoRI* site. The pMSCV-neo-FLAG-MLL-ENL, pMSCV-neo-FLAG-MLL-AF4 and pMSCV-FLAG-MLL-AF4 constructs were made by Dr. Inusha de Silva of the MHCB unit. All three of these constructs contained 1 FLAG tag at the 5' end of MLL-fusion. The pMSCV-neo-TRE construct was made by Dr. Michelle Morrow of the MHCB unit.

2.2 Culture of LinXE and NIH3T3 fibroblast cell lines

The LinXE ecotropic retrovirus packaging cell line, 293T and NIH3T3 cell lines were cultured in complete medium (Dulbecco's Modified Eagle's medium [DMEM, Invitrogen] supplemented with 10% foetal calf serum [FCS] and 2 mM L-glutamine). The LinXE cells were maintained in 7.5 μg/ml hygromycin (Cayla) to select for the presence of the gag, pol and env genes.

2.3 Transfection of LinXE cells

LinXE cells were seeded at a density of 0.75 x 10^6 per 10cm plate 3 days prior to transfection. Cells were transfected with 8 μg retroviral plasmid DNA using 40 μl Lipofectamine (Invitrogen) in a total volume of 8 ml optimum (Invitrogen) according to the manufacturer's instructions.

2.4 Retroviral transduction of NIH3T3 cells

NIH3T3 cells were seeded the day before transduction at a density of 2 x 10^5 per 6cm plate. Retroviral supernatant, which was harvested from LinXE cells 48 hours after transfection, was cleared of cell debris using a 0.45μm filter. NIH3T3 cells were transduced by replacing their media with cleared retroviral supernatant diluted 1:4 in complete medium supplemented with 5 μg/ml polybrene (Sigma-Aldrich). The NIH3T3 cells were then returned to culture overnight. For the co-transductions,

equal volumes of the two retroviral supernatants were mixed and diluted 1:2 in complete medium. After 24 hours the media was changed. The cells were analysed for viral protein expression by flow cytometry or western blot 48 hours after transduction.

2.5 Determination of viral titre by titration on NIH3T3 cells

NIH3T3 cells were seeded the day before transduction at a density of 5 x 10^4 cells per well of a 6-well plate. Viral supernatant was harvested from LinXE cells as previously described and diluted 1:10, 1:1000 or 1:100000 in complete medium. NIH3T3 cells were transduced with 2 ml of each virus dilution in the presence of 5 μg/ml polybrene. The cells were passaged after 24 hours and were reseeded at a 1:20 dilution into 10cm plates. Antibiotic selection was added to the cells 48 hours after transduction. 1 mg/ml G418 (Invitrogen) was used to select for cells transduced with pMSCV-neo and 250 μg/ml hygromycin (Cayla) was used to select for cells transduced with pREV-TRE. Fresh media and selection was added to the plates 5 days after selection was initiated. The number of colonies on each plate was counted 12 days after transduction. In order to count the colonies, the plates were washed in PBS and the colonies were visualised by staining with 1.7 mg/ml methylene blue (Sigma-Aldrich) in Methanol. The viral titre was calculated using the following formula:

$$\text{Viral titre (infectious particles / ml)} = \frac{\text{number of colonies x dilution factor}}{2}$$

2.6 Isolation of haematopoietic progenitor cells (HPCs)

HPCs were isolated from mice which were maintained in the animal facilities of the National Institute for Medical Research. All experiments were performed according to institutional guidelines and Home Office regulations.

i) Foetal HPCs

c-Kit$^+$ Ter-119$^-$ or c-Kit$^+$ Sca-1$^+$ HPCs were purified by Dr. Owen Williams from embryonic day 12 (E12) foetal liver of C57BL/10 mice by fluorescence activated cell sorting (FACS) using a MoFlo sorter (DakoCytomation) and monoclonal antibodies (mAb) specific to c-Kit and Ter-119 or Sca-1 (Table 2.2).

ii) Adult HPCs

Bone marrow was extracted by Dr. Owen Williams from 4-6 week old C57BL/10 or C57BL/6 mice 5 days after the injection of 150 mg/kg of 5-fluorouracil (5-FU) into the lateral tail vein. A single cell suspension was made from the marrow of the femur and tibia and the red cells were lysed in 2 ml of erythrocyte lysis buffer for 10 minutes at room temperature. In some experiments HPCs were purified by magnetic activated cell sorting (MACS) using MACS separation columns (Miltenyi Biotech) and monoclonal antibodies specific to c-Kit (Table 2.2). In other cases, lineage depleted cells were isolated by MACS using a lineage panel kit which consisted of α CD3 (145-2C11), α Mac-1 (M1/70), α B220 (RA3-6B2), α Gr-1 (RB6-8C5), α Ter-119 (BD Pharmingen).

Foetal HPCs were seeded at a density of 2 x 10^5/ml and unsorted adult HPCs were seeded at a density of 1 x 10^6/ml. HPCs were stimulated overnight in complete medium supplemented with 50 μM 2-mercaptoethanol (2-ME) (BDH), 100 ng/ml stem cell factor (SCF), 10 ng/ml interleukin-6 (IL-6) and 10 ng/ml interleukin-3 (IL-3). In some experiments the complete medium was supplemented with 20 ng/ml interleukin-7 (IL-7) and 10 ng/ml *fms*-like tyrosine kinase-3 ligand (Flt-3L), instead of IL-3 and IL-6. All recombinant murine growth factors were supplied by Peprotech EC.

2.7 Transduction of haematopoietic progenitor cells

Retroviral supernatant was harvested as described previously. HPCs were transduced on two consecutive days. Complete medium was added to the LinXE cells after the first virus harvest and fresh virus was collected from the same culture the following day. The retroviral supernatant was concentrated 10-fold in some experiments. The supernatant was cleared of cell debris by 2 rounds of centrifugation at 580g for 5 minutes. Cleared virus was then aliquotted into 1.5ml eppendorf tubes and concentrated by centrifugation for 1 hour at 16,000g. HPCs were transduced at a density of 10^4 cells per well of a 96-well flat-bottomed plate. A total of 6 wells were set up for each condition and 6 x 10^4 HPCs were transduced with a total of 600 μl virus unless otherwise stated. The viral supernatant was supplemented with 20% FCS, 50 μM 2-ME, 5 μg/ml polybrene, 100 ng/ml SCF, 10

ng/ml IL-3 and 10 ng/ml IL-6. In some experiments the cells were stimulated prior to transduction with SCF, IL-7 and Flt-3L (section 2.6). In these experiments, the viral supernatant was supplemented with 50 μM 2-ME, 5 μg/ml polybrene, 100 ng/ml SCF, 20 ng/ml IL-7 and 10 ng/ml Flt-3L. The cells were transduced by spinoculation (centrifugation at 700 g, 25°C, 45 minutes) and then returned to culture. The transduction was repeated after 24 hours, 60 μl of medium was removed from each well and replaced with 100 μl of fresh viral supernatant and growth factors. After culture overnight, the transduced HPCs were used for colony forming assays.

2.8 Colony forming cell assays

Approximately 24 hours after the second transduction, the 6 wells of transduced cells were pooled, washed in 5 ml Minimal Essential-Alpha Media (MEM) (Invitrogen) and resuspended in 100 μl MEM. For the myeloid assays the cells were added to 3 ml of methylcellulose (M3434) (Stem cell Technologies Inc.), containing IL-3, IL-6 and SCF which was supplemented with 10 ng/ml GM-CSF (Peprotech). For the B cell assays, the cells were added to 2.4 ml methylcellulose (M3231) (Stem cell Technologies Inc.) which was supplemented with 100 ng/ml SCF, 20 ng/ml IL-7 and 10 ng/ml Flt-3L. Selection was employed in the first round of methylcellulose plating. 1 mg/ml G418 (Invitrogen) was used to select for cells transduced with pMSCV-neo constructs and 1.5 mg/ml hygromycin (Cayla) was used to select for cells transduced with pREV-TRE constructs. The volume of methylcellulose containing the cells, antibiotic and growth factors was made up to 3.3 ml with MEM. The components were then mixed thoroughly and 1.1 ml was aliquotted into duplicate 35mm plates according to the manufacturer's instructions. Duplicate methylcellulose cultures were placed in 10cm plates and cultured for 6-10 days alongside a 35mm plate containing PBS to humidify the cultures.

After 6-10 days, colonies containing 50 cells or more were scored. The cells were harvested from the methylcellulose by the addition of 1 ml MEM to each plate, followed by gentle pipetting to create a single cell suspension. The cells from duplicate methylcellulose cultures were pooled and washed in 10 ml MEM containing 2% FCS. The cells were counted and 1 x 10^4 cells were replated into

secondary assays under identical conditions but in the absence of antibiotic selection. This process was repeated every 7-10 days. In some cases 2 μg/ml doxycycline (BDH) was added in subsequent rounds. Colonies were stained with 1 mg/ml p-iodonitrotetrazolium (INT) (Sigma-Aldrich) in PBS.

2.9 Generation of cell lines in liquid culture

Cells harvested from the fourth round of the myeloid colony forming assays were seeded at a density of 1 x 10^5 cells/ml in RPMI-1640 (Invitrogen) with 10% FCS, 2 mM L-glutamine and 50 μM 2-ME supplemented with 100 ng/ml SCF, 10 ng/ml IL-6 and 10 ng/ml IL-3. Cells harvested from the fourth round of the B cell colony forming assays were seeded at a density of 1 x 10^5 cells/ml in RPMI-1640 with 10% FCS, 2 mM L-glutamine and 50 μM 2-ME supplemented with 100 ng/ml SCF, 20 ng/ml IL-7 and 10 ng/ml Flt-3L.

2.10 Flow cytometry

Cells were washed in PBS / 0.05% sodium azide and pre-incubated with 100 μl stain buffer containing unlabelled anti-Fcγ III/II Receptor mAb for 15-30 minutes. Following this blocking step, the cells were washed and each subsequent stain was performed for 30 minutes in a total volume of 100 μl stain buffer containing the appropriate antibody. For the reporter gene experiments, NIH3T3 cells or HPCs were stained with biotin-conjugated mAb specific for CD2. In other experiments, HPCs were stained with phycoerythrin (PE)-, allophycocyanin (APC)-, biotin- or fluorescein isothiocyanate (FITC)- conjugated mAbs specific for various markers of cellular differentiation (Table 2.2). Biotin-conjugated mAb were visualised using Streptavidin-PE or Streptavidin-Tricolour (Streptavidin-TC). Flow-cytometry was performed using either a Beckman Coulter Epics XL analyser and EXPO3 software (Beckman Coulter) or a Cyan ADP analyser and Summit 4.1 software (DakoCytomation).

Table 2.2 Flow cytometry antibodies used in this study.

Antibody	Clone	Isotype	Supplier	Working dilution
Anti- Fcγ III/II	2.4G2	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Anti-CD2 Bio	LFA2	$IgG_{2b\,\lambda}$	BD Pharmingen	1:100
Anti-cKit PE	2B8	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Anti-cKit FITC	2B8	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Anti-Sca-1 PE	D7	$IgG_{2a\,\kappa}$	BD Pharmingen	1:100
Anti-Mac-1 PE	M1/70	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Anti-Mac-1 FITC	M1/70	$IgG_{2b\,\kappa}$	eBioscience	1:100
Anti-Gr-1 PE	RB6-8CS	$IgG_{2b\,\kappa}$	BD Pharmingen	1:200
Anti-Gr-1 APC	RB6-8CS	$IgG_{2b\,\kappa}$	eBioscience	1:100
Anti-CD43 PE	S7	$IgG_{2a\,\kappa}$	BD Pharmingen	1:500
Anti-Ter119 PE	Ter119	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Anti-F4/80 PE	F4/80	$IgG_{2a\,\kappa}$	Caltag	1:100
Anti-B220 PE	RA3-6B2	$IgG_{2a\,\kappa}$	BD Pharmingen	1:100
Anti-CD19 PE	1D3	$IgG_{2a\,\kappa}$	BD Pharmingen	1:100
Anti-BP1 Bio	6C3	$IgG_{2a\,\kappa}$	eBioscience	1:100
Anti-CD45.2 Bio	104	$IgG_{2a\,\kappa}$	eBioscience	1:100
Anti-IgG2A PE	G155-178	$IgG_{2a\,\kappa}$	BD Pharmingen	1:100
Anti-IgG2B PE	MPC-11	$IgG_{2b\,\kappa}$	BD Pharmingen	1:100
Streptavidin PE	N/A	N/A	BD Pharmingen	1:250
Streptavidin TC	N/A	N/A	Caltag	1:100

2.11 *In vivo* leukaemogenicity assays

NOD/SCID mice were maintained in the animal facilities of the Institute of Child Health and all experiments were performed according to institutional guidelines and Home Office regulations. 10^6 immortalised cells were resuspended in 300 µl of Hanks balanced saline solution (HBSS) (Invitrogen) buffered with 10 mM Hepes (Invitrogen). The cells were then injected into the intraperitoneal cavity of 6-8 week old NOD/SCID mice by Dr. Owen Williams. The peripheral blood of these mice was analysed for the presence of leukaemic cells at regular intervals two months

after transfer. Approximately 100 µl of peripheral blood was collected by Dr. Owen Williams from the lateral tail vein following a small incision and mixed immediately with 20 USP units/ml of Heparin (Sigma Aldrich) to prevent it from clotting. The red cells were lysed in erythrocyte lysis buffer, washed with PBS / 0.05% sodium azide and stained with the Mac-1-PE-conjugated mAb (Table 2.2). Mice were sacrificed when they showed more than two moderate symptoms of illness such as weight-loss, marked piloerection, subdued behaviour or hunched posture. Single cell suspensions were then made from their bone marrow, spleen and lymph nodes. In some cases, 2 x 10^6 to 10^7 splenocytes were resuspended in 200 µl HBSS / 10 mM Hepes and injected into the intraperitoneal cavity of secondary recipients. Approximately 500 µg of dox was injected into the intraperitoneal cavity of some of the primary recipient mice by Dr. Owen Williams. The following day these mice were administered 2 mg/ml dox and 2.5% sucrose in the drinking water.

2.12 Cytokine induced differentiation of the cell lines

Differentiation of the cell lines was performed in RPMI-1640 with 10% FCS, 2 mM L-glutamine and 50 µM 2-ME supplemented with either 10 ng/ml granulocyte colony-stimulating factor (G-CSF) or 10 ng/ml macrophage colony-stimulating factor (M-CSF).

2.13 Cytopsin analysis of cellular morphology

Approximately 3 x 10^4 cells were washed in PBS and resuspended in 100 µl PBS. Cells were centrifuged onto slides at 35g for 5 minutes at low deceleration using a cytospin 3 machine (Shandon). The slides were then fixed and stained with May-Grunwald-Giemsa (MGG) using a Shandon varistain 24-4 automated staining machine in the Haematology department at Great Ormond Street Hospital.

2.14 Histological analysis of murine tissue

The liver and kidney from moribund mice were fixed in formalin (Sigma Aldrich). These tissues were then sectioned and stained with hematoxylin and eosin by Dr. Neil Sebire of the histopathology department at Great Ormond Street Hospital.

2.15 MTS assay

Cells were seeded at a density of between 10^3 to 10^4 cells per well of a flat-bottomed 96-well plate depending on the rate of proliferation of the cell line. The cells were cultured in RPMI-1640 with 10% FCS, 2 mM L-glutamine and 50 μM 2-ME supplemented with various combinations of cytokines. These included 100 ng/ml SCF, 10 ng/ml IL-3, 10 ng/ml IL-6, 10 ng/ml GM-CSF, 10 ng/ml G-CSF, 10 ng/ml M-CSF, 20 ng/ml IL-7 and 10 ng/ml Flt-3L. After a specified period of culture, CellTiter 96® Aq_{ueous} One Solution Reagent (Promega) was added to each well according to the manufacturer's instructions. The plates were wrapped in foil and returned to culture for 4 hours. After this time the cell viability was measured by reading the absorbance at 490nm using a 550 Biorad plate-reader.

2.16 Preparation of cellular extracts for Western blot

Adherent cells from confluent 6cm plates and 1-5 x 10^6 immortalised cells were washed in PBS and then lysed on ice for 10 minutes. Adherent cells were lysed using either 240 μl RIPA buffer containing 10 μg/ml Aprotinin, 250 μg/ml PMSF, 10 μg/ml Leupeptin and 10 μg/ml Pepstatin A or 240 μl NP40 buffer containing 1 x complete protease inhibitor tablets (Roche). Immortalised cells were lysed using 60 μl NP40 buffer containing 1 x protease inhibitor tablets. DNA was pelleted by centrifugation at 16000g for 10 minutes at 4°C. The supernatant containing the protein extract was mixed with 1 x sample buffer and then the lysate was snap-frozen and stored at -20°C until it was analysed by Western blot.

2.17 Western blot analysis of protein expression

Lysates were boiled at 100°C for 5 minutes and then 20μl was loaded onto 6 % SDS-polyacrylamide gels along with 10 μl Rainbow™ molecular weight marker (Amersham Pharmacia). The 6% resolving gel and 4% stacking gel were prepared according to standard protocols. Electrophoresis was performed for 16 hours at 40V in 1 x running buffer. Protein was then transferred to a PVDF membrane (Immobilon-P, Millipore) for 5 hours at 0.5A in 1 x transfer buffer. Membranes were blocked in PBS with 5% non-fat milk and 0.2% tween-20. MLL-ENL fusion proteins were visualised by staining with anti-myc, anti-HA, anti-FLAG or anti-MLL antibodies (Table 2.3). Proteins were detected using the appropriate

secondary horse radish peroxidase (HRP)-conjugated antibody (Table 2.3), and a chemiluminescent Reagent (ECL, Amersham Biosciences) according to the manufacturer's instructions. Membranes were stripped in 1 x stripping buffer, the blocking step was repeated and the membranes were re-probed with anti-alpha tubulin as a loading control.

2.18 Isolation of genomic DNA from cells

5-10 x 10^6 cells were washed in PBS and lysed overnight in 400 µl TNES containing 0.5 mg/ml proteinase K (Roche) at 37°C. Genomic DNA was extracted using Phenol-Chloroform-Isoamylalcohol (Sigma-Aldrich). The lysate was mixed with 0.5 ml of Phenol-Chloroform-Isoamylalcohol (25:24:1) by shaking vigorously for 1 minute. The phases were separated by centrifugation at 16000g for 5 minutes and the supernatant was transferred to a tube containing 1 ml ethanol:3M NaOAc (25:1). The DNA was precipitated by freezing on dry ice for 2 hours, pelleted by centrifugation at 16000g for 10 minutes and then washed in 70% ethanol. The DNA was vacuum dried and dissolved in 100 µl TE (pH 8.0).

Table 2.3 Western antibodies used in this study.

Antibody	Clone	Supplier	Working dilution
Mouse anti-Myc	9E10	Roche	1:400
Mouse anti-Myc	9B11	Cell signalling technologies	1:1000
Mouse anti-Flag	M2	Sigma Aldrich	1:800
Rat anti-HA	3F10	Roche	1:500
Mouse anti-MLL	N4.4	Dr. M.L Cleary, Stanford.	1:3
Rat anti-tubulin	YL1/2	Serotec	1:1000
Sheep anti-mouse HRP	N/A	Amersham Biosciences	1:2000
Sheep anti-rat HRP	N/A	Serotec	1:2000

2.19 Southern blot analysis of DNA

Southern blotting was performed according to standard protocols. Briefly, 10 µg of genomic DNA was digested with 44 units *EcoRI* in the presence of 40 µg/ml BSA

and 10 μg RNase overnight. Digested DNA was resolved on a 0.8% agarose gel for 16 hours at 35V. The DNA was denatured by soaking the gel in denaturing solution for 45 minutes. Following this, the gel was washed in water, soaked in neutralising solution for 40 minutes and then washed in 10 x SSC (National Diagnostics). DNA was transferred onto a nylon-based membrane (genescreen, NEN Lifescience) overnight by capillary transfer according to standard wet-blotting protocols. The membrane was then baked for 2 hours at 80°C in order to fix the DNA onto the membrane.

A hybridization probe was prepared by digesting pCSARQ2 with *HindIII*. This digest released a 2.1 kb cDNA fragment which contains N-terminal MLL sequence. This fragment was isolated and gel purified for use in probing the membrane. The probe (100 ng) was denatured at 94°C for 10 minutes and then labelled with [^{32}P] α-dCTP using random primer labelling. The components of the labelling reaction were 1 x labelling buffer, 100 ng hexanucleotide primers (Sigma-Aldrich), 0.4 mg/ml BSA, 20 mM $MgCl_2$, 5 units Klenow (Promega), 100 ng denatured probe and 1.11 MBq ^{32}P dCTP. The labelling reaction was carried out at 37°C for 2 hours and then stopped by the addition of 0.1% SDS in TE. The probe was purified using Sephadex G50 columns equilibrated with 0.1 M EDTA. The probe was eluted from the column by centrifugation at 390g for 3 minutes into 1 M NaOH to denature the probe. The probe was then neutralised by the addition of 2 M Tris (pH 8.5).

The membrane was blocked prior to hybridisation by incubation with pre-hybridisation solution containing 0.5 mg/ml salmon sperm DNA (Sigma-Aldrich) for 2 hours at 65°C. After this time, the probe was added and allowed to hybridise to the membrane overnight at 65°C. The membrane was then washed twice in 2 x SSC / 0.1% SDS at 55°C and bands were visualised using a Typhoon phosphorimager (Amersham Biosciences).

2.20 Isolation of total RNA from cells

3-6 x 10^6 cells were lysed in 1 ml of Trizol (Invitrogen). The lysate was mixed with 0.2 ml chloroform by shaking vigorously for 15 seconds and then incubated at room temperature for 15 minutes. Phases were separated by centrifugation at 12000g for

15 minutes at 4°C. The upper aqueous phase was isolated, mixed with 0.5 ml isopropanol and incubated at room temperature for 10 minutes. The RNA was precipitated by centrifugation at 12000g for 10 minutes at 4°C and washed in 70% ethanol. The RNA was then air-dried and re-suspended in 30 µl diethyl polycarbonate (DEPC) treated H_2O (Invitrogen).

2.21 cDNA synthesis

4 - 10 µg of RNA was treated with 2 units of DNase I (Invitrogen) for 15 minutes at 22°C to degrade genomic DNA. The DNase was then neutralised using 2.5mM EDTA (Invitrogen) and heat inactivated at 65°C for 5 minutes. Reverse transcription was performed using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) at a concentration of 10^5 U/mL, in the presence of 0.5 mM dNTP, 5 x 10^3 U/mL RNase Inhibitor and 50 µM random hexamer primers (all Invitrogen) at 37°C for two hours. Negative controls contained equimolar quantities of all the reactants except the MMLV-RT enzyme.

2.22 Polymerase Chain Reaction (PCR)

The MLL-ENL transcript was detected by PCR amplification of cDNA using primers that span the MLL-ENL breakpoint. Two MLL primers and 1 ENL primer were designed:

MLL forward primer 1: 5' CAGAATCTACAATGGATGCC 3'

MLL forward primer 2: 5' CCGCCAAGAAAAGAAGTTCCC 3'

ENL reverse primer: 5' GGACAAACACCATCCAGTCG 3'.

The first MLL primer and the ENL primer generate a PCR product of 846 bp. These primers were used in chapter 4. The second MLL primer and the ENL primer generate a PCR product of 490 bp. These primers were used in chapter 5.

Other primers used were:

GM-CSFR forward primer: 5' GGAGACCCGCCTCGCCTTCC 3'

GM-CSFR reverse primer: 5' ACTCGCACGTCGTCGGACAC 3'

G-CSFR forward primer: 5' ACAGGAGTGTGAACTTCGCT 3'

G-CSFR reverse primer: 5' TTGCTTCTTCTGACACCACG 3'

M-CSFR forward primer: 5' ATGAGTCCCTCTTCACTCCG 3'

M-CSFR reverse primer: 5' ACCTTCAGCACTGCATCTTC 3'

PCR was performed using 250 ng cDNA, 0.625 units Taq DNA polymerase (AB gene), 0.2 mM dNTPs (Ammersham Pharmacia), 1.5 mM $MgCl_2$ and 25 pmol of each primer. The reaction was comprised of 35 cycles. An initial denaturation step was performed at 94°C for 5 minutes. Each cycle was composed of three steps: a denaturation step at 94°C for 30 seconds, an annealing step at 50°C for 1 minute and an elongation step at 72°C for 1 minute.

2.23 Real-time Quantitative PCR (Q-PCR)

Q-PCR was carried out using TaqMan™ probe-based chemistry (Applied Biosystems). This chemistry utilizes a fluorogenic oligonucleotide probe consisting of a 5' reporter and a 3' quencher that anneals specifically between the forward and reverse primers. Global *Hox* gene expression profiling was carried out by D. Grier, G. McGonigle, A. Thompson and T. Lappin, Queen's University, Belfast. This analysis allowed the absolute *Hox* gene copy number to be determined. In other experiments, pre-designed probe and primer sets (Applied Biosystems) were used to examine the relative expression levels of the Hox co-factor genes and *Flt-3*. All data was normalised to 18S rRNA expression. Quadruplicate Q-PCR reactions were carried out in 96-well plates in a total volume of 20 µl using 5 ng cDNA, 1 x TaqMan fast universal PCR mastermix (Applied Biosystems) and 1 x probe / primer set. An initial incubation at 95°C for 20 seconds activated the Amplitaq Gold™ DNA polymerase. The amplification reaction consisted of 40 cycles and each cycle was composed of 2 steps, a denaturation step at 95°C for 1 second and an annealing / extension step at 60°C for 20 seconds. The amplifications were performed using an ABI Prism 7900HT fast Sequence Detection System (Applied Biosystems) and data was analysed using Sequence Detector v 2.2.2 software (Applied Biosystems).

2.24 The design of a Q-PCR assay to determine MLL-ENL transcript expression

In order to measure MLL-ENL transcript expression by Q-PCR, two primers and a probe were designed using Primer express software (Applied Biosystems). The primers flanked the MLL-ENL breakpoint and the probe was designed such that it spanned the breakpoint:

Forward primer: 5' CAGGGTGGTTTGCTTTCTCTGT 3'

Reverse primer: 5' GCGATGCCCCAGCTCTAA 3'

Probe: 5' TGGACGGTGCACTCTACATGCCCACTA 3'

These sequences were submitted to Applied Biosystems who then synthesised the primers and probe. The 5' reporter of the probe was 6-carboxyfluorescein (FAM) and the 3' quencher was 6-carboxy-tetramethylrhodamine (TAMRA). In order to determine the optimum concentration of each primer, Q-PCR reactions were set up using various combinations of concentrations of the two primers (50 nM to 900 nM). The reactions were carried out using 50 ng cDNA (prepared from total RNA isolated from TRE-ME2), 50 nM of probe and 1 x TaqMan universal PCR mastermix. An initial incubation step at 50°C allowed uracil-N-glycosylase (UNG) to remove contaminating PCR products. This was followed by incubation at 95°C for 10 minutes to inactivate the UNG enzyme and activate the Amplitaq Gold™ DNA polymerase. The amplification reaction consisted of 40 cycles and each cycle was composed of 2 steps, a denaturation step at 95°C for 15 seconds and an annealing / extension step at 60°C for 1 minute. All optimisation reactions were carried out in quadruplicate using the ABI 7000 Sequence Detection System and associated software (version 1.2) (Applied Biosystems). The results of this optimisation step are shown in the appendix (Figure A.1A). During the exponential phase of the amplification, a fluorescence signal threshold is set at which point all the reactions can be compared. The threshold cycle (C_T) value is the number of PCR cycles required to generate sufficient fluorescent signal to reach this threshold (Ginzinger, 2002). The fluorescent signal (Rn) value is greatest at the end of the exponential phase of the amplification. Hence, the longer the exponential phase of the reaction, the greater the accumulation of fluorescent signal and the higher the Rn value. The optimum primer concentrations were 900 nM of the forward primer (900f) and 300 nM of the reverse primer (300r). This combination was optimal since it produced the lowest C_T value (Figure A.1A) and the highest Rn value (data not shown).

Having optimised the primer concentrations, the optimum probe concentration was determined by setting up Q-PCR reactions using different concentrations of probe (25 nM to 225 nM). The reactions were carried out using 50 ng cDNA, 900 nM of the forward primer, 300 nM of the reverse primer and 1 x TaqMan universal PCR mastermix. The results of this optimisation step are shown in the appendix (Figure

A.1B). The optimum concentration of probe was 150 nM since this was the lowest concentration used which did not affect the C_T value.

In order to examine whether the Q-PCR reaction was efficient at low and high cDNA concentrations, a standard curve was generated. Q-PCR reactions were set up using various concentrations of cDNA (3.125 ng to 100 ng) in the presence of 900 nM of the forward primer, 300 nM of the reverse primer, 150 nM probe and 1 x TaqMan universal PCR mastermix. In order to analyse the MLL-ENL Q-PCR data using the $2^{-\Delta\Delta CT}$ relative quantitation method (Livak and Schmittgen, 2001), it is important to ensure that the MLL-ENL and GAPDH (endogenous control) Q-PCR reactions are equally efficient. Therefore, a standard curve was also generated for GAPDH. Reactions were set up using various concentrations of cDNA (3.125 ng to 100 ng), 1 x GAPDH probe and primer set (optimised by the manufacturer [Applied Biosystems]) and 1 x Taqman universal PCR mastermix. The MLL-ENL and GAPDH standard curves are shown in the appendix (Figure A.2). The correlation co-efficients of the MLL-ENL and GAPDH standard curves were 0.9969 and 0.9961, respectively. Therefore, the MLL-ENL and GAPDH Q-PCR assays are reproducible at high and low cDNA concentrations. The efficiency of each reaction was calculated using the formula:

PCR efficiency = $(10^{(1/-s)})$-1 (Ginzinger *et al*, 2002), where s is the gradient of the standard curve. The efficiency of the MLL-ENL reaction was 103% and that of GAPDH was 109%. Since the efficiencies were comparable, the $2^{-\Delta\Delta CT}$ relative quantitation method was used to determine the relative level of MLL-ENL expression.

2.25 Affymetrix gene chip arrays

RNA was prepared from 3 conditional cell lines and 1 constitutive cell line following treatment with or without dox. The integrity of the RNA was determined using the Agilent 2100 Bioanalyser (Agilent Technologies) according to the manufacturer's instructions. The ratio of 28S:18S ribosomal RNA in each sample was between 1.6 and 2. This indicates that the RNA was not degraded and was of a high quality. A representative electopherogram and densitometry plot is shown in Figure A.3. The concentration and purity of the RNA were determined using the ND-1000 spectrophotometer (Nanodrop). The A_{260}:A_{280} ratio of each RNA sample was between 1.8 and 1.9. Therefore, the RNA samples were of a sufficiently high

quality and purity to proceed with the cDNA synthesis reaction. cDNA was synthesised using the Roche Microarray cDNA synthesis kit according to the manufacturer's instructions. All subsequent steps were performed using Affymetrix reagents according to the instructions in the Affymetrix expression analysis technical manual. Briefly, 5 μg of RNA was reverse transcribed using a T7-Oligo(dT) promoter primer in the first-strand cDNA synthesis reaction. The second strand cDNA synthesis reaction was then carried out following the removal of hybridised RNA using RNase H. The double-stranded cDNA product was purified and used as a template for the *in vitro* transcription (IVT) reaction. Biotinylated complimentary RNA (cRNA) was amplified in the IVT reaction using T7 RNA polymerase and a biotinylated ribonucleotide analogue mix. The biotinylated cRNA was then purified, fragmented and hybridised to GeneChip® Mouse Genome 430 (version 2) arrays (Affymetrix). Following hybridisation, the arrays were washed and stained with streptavidin phycoerythrin using a Fluidics Station (FS450) (Affymetrix). The fluorescence was amplified by adding a biotinylated anti-streptavidin antibody and more streptavidin phycoerythrin. The arrays were then scanned following excitation at 570 nm using a GeneChip Scanner (GCS3000) (Affymetrix). This process is summarised in Figure 2.3.

Affymetrix microarray suite 5 (MAS5) was used to quantitate expression levels for each gene. The arrays are designed such that for every probe on the array, there is also a mismatch probe that differs from the perfect match probe by one nucleotide. By comparing the level of hybridisation of the perfect match and the corresponding mismatch probes to the target sequence, it was possible to assign a detection call of present or absent for each gene. Since there are multiple probe pairs within each probe set, a detection algorithm was used to generate a detection p value which reflects the confidence of the detection call for that probe set. A signal value was then determined for each gene after correction for non-specific signal by subtraction of the mismatch probe value. In order to compare gene signals across multiple arrays, a scaling factor was determined for each array. The total signal of each array was averaged and the scaling factor required to adjust the average signal of each array to an arbitrary target of 100 was calculated. The signal values of each array were then multiplied by the appropriate scaling factor. The scaling factor of each array was within 3 standard deviations of the mean. Therefore, gene signals across

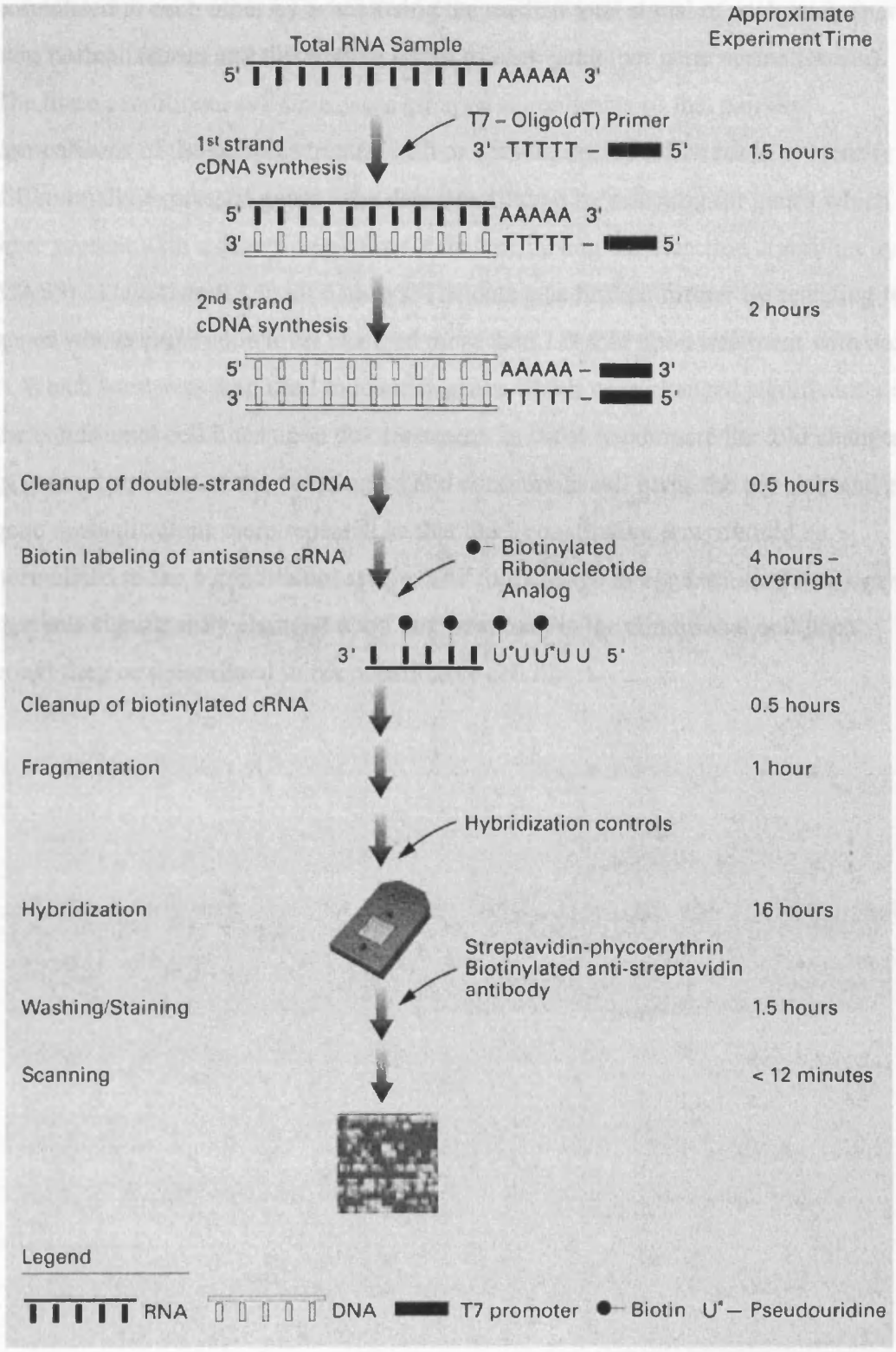

Figure 2.3 Overview of the steps undertaken to analyse gene expression by microarray. Adapted from the Affymetrix expression analysis technical manual.

each array could be directly compared. The data was analysed using GeneSpring version 7.2 software (Agilent Technologies). The 6 conditional arrays were normalised to each other by normalising the median total signal of each array (per chip normalisation) and the median signal of each gene (per gene normalisation). The three conditional cell lines were grouped as replicates so that pairwise comparisons of the samples treated with or without dox could be made to identify differentially expressed genes. The data was filtered by selecting for genes which were present with a detection p-value (calculated using the detection algorithm by MAS5) of less than 0.1 in all 6 arrays. The data was filtered further by selecting for genes whose expression level changed more than 1.3-fold upon treatment with dox. A Welch t-test was then used to identify genes which were changed significantly in the conditional cell lines upon dox treatment. In order to compare the fold changes in gene expression of the constitutive and conditional cell lines, the per chip and per gene normalisations were repeated so that the 2 constitutive arrays could be normalised to the 6 conditional arrays. The fold change in expression of every gene that was significantly changed upon dox treatment in the conditional cell lines could then be determined in the constitutive cell line.

Chapter 3 Delivery of two retroviral constructs that enable conditional protein expression in target cells.

The Tet-Off conditional expression system is comprised of two components. The first component is the tetracycline transactivator (tTA) which is a fusion of the Tet repressor (TetR) and the activation domain of the herpes simplex virus VP16 protein. The second component is the tetracycline response element (TRE) which is upstream of the gene to be over-expressed. The TRE contains seven direct repeats of the tetracycline resistance operon (*TetO*), upstream of a minimal CMV promoter which can be bound by the tTA (Gossen and Bujard, 1992).

In order to assess the efficacy of using two retroviral vectors to deliver this expression system to target cells, pMSCV-tTA-IRES-EGFP and pREV-TRE-hCD2t retroviral reporter constructs were made. The internal ribosome entry site (IRES) allows the translation of two proteins from a bicistronic mRNA. Since tTA is linked via an IRES to the enhanced green fluorescent protein (EGFP), target cells successfully transduced with the tTA expression vector can be identified based on their EGFP expression profile. In the absence of doxycycline (dox), tTA binds to the TRE and activates transcription of the human tail-less CD2 (hCD2t) gene (Figure 3.1). In the presence of dox, the antibiotic binds to tTA and causes a conformational change such that tTA is no longer able to bind to the TRE and activate transcription of the CD2 gene. These reporters were used to optimise dual transduction of NIH3T3 fibroblast cells and ultimately haematopoietic progenitor cells (HPCs), the target cells for transformation with MLL-ENL expression constructs.

3.1 Optimisation of NIH3T3 cell transduction with two retroviral constructs.

A series of NIH3T3 transductions were performed in order to determine the optimum conditions for dual transduction with two retroviral reporter constructs. NIH3T3 cells were transduced with pMSCV-tTA-IRES-EGFP and then at various time points after the initial transduction (6 hours, 19 hours and 29 hours), with pREV-TRE-hCD2t. In addition, cells were simultaneously co-transduced with both retroviral constructs. The optimum transduction conditions were determined by

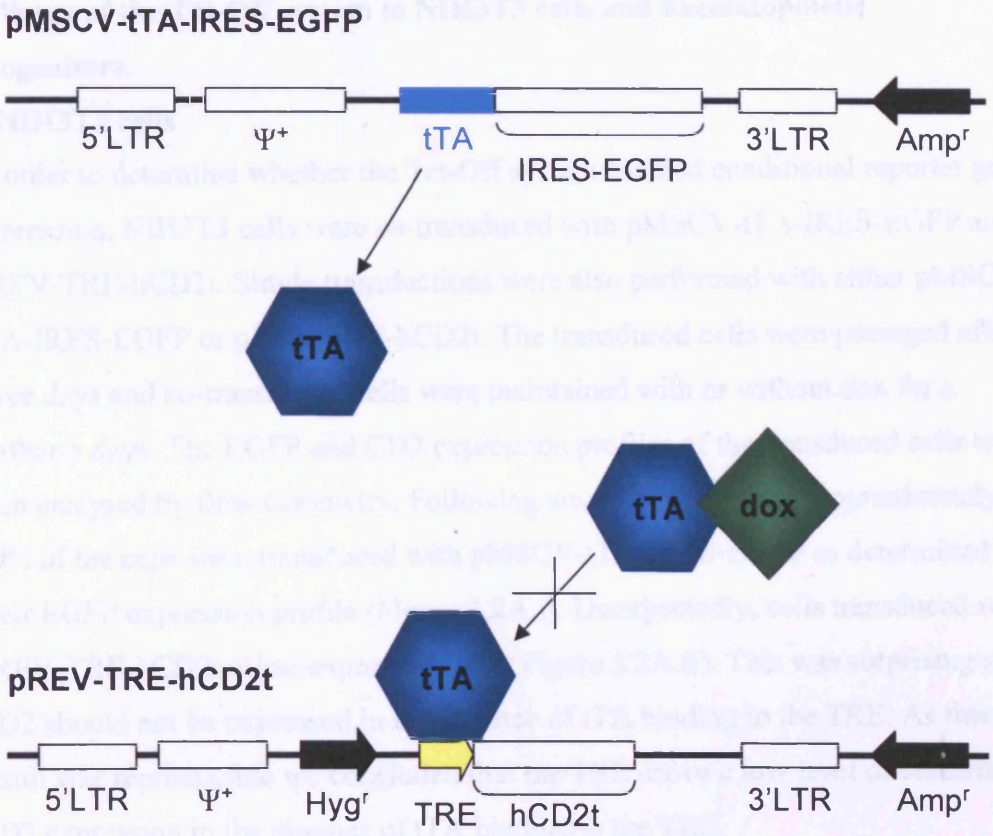

Figure 3.1 The use of two retroviral reporter constructs to deliver the Tet-Off system to target cells. In the absence of doxycycline (dox) tTA binds to the TRE and activates expression of the CD2 gene. In the presence of dox tTA undergoes a conformational change such that it is no longer able to bind to the TRE and therefore CD2 is not expressed. LTR: long terminal repeat, ψ^+: viral packaging signal, tTA: tetracycline transactivator, Amp^r: ampicillin resistance gene, Hyg^r: hygromycin resistance gene, IRES: internal ribosome entry site, EGFP: enhanced green fluorescent protein, hCD2t: human tail-less CD2.

measuring EGFP and CD2 expression approximately 48 hours after the second transduction was performed, by flow cytometry. Maximum co-expression of EGFP and CD2 was obtained when the cells were simultaneously transduced with both retroviral constructs (data not shown).

3.2 Conditional reporter gene expression can be achieved following retroviral delivery of the Tet-Off system to NIH3T3 cells and haematopoietic progenitors.

i) NIH3T3 cells

In order to determine whether the Tet-Off system enabled conditional reporter gene expression, NIH3T3 cells were co-transduced with pMSCV-tTA-IRES-EGFP and pREV-TRE-hCD2t. Single transductions were also performed with either pMSCV-tTA-IRES-EGFP or pREV-TRE-hCD2t. The transduced cells were passaged after three days and co-transduced cells were maintained with or without dox for a further 5 days. The EGFP and CD2 expression profiles of the transduced cells were then analysed by flow cytometry. Following single transductions, approximately 30% of the cells were transduced with pMSCV-tTA-IRES-EGFP as determined by their EGFP expression profile (Figure 3.2A.i). Unexpectedly, cells transduced with pREV-TRE-hCD2t alone expressed CD2 (Figure 3.2A.ii). This was surprising since CD2 should not be expressed in the absence of tTA binding to the TRE. As this result was reproducible we concluded that the TRE drove a low level of constitutive CD2 expression in the absence of tTA binding to the TRE.

Following co-transduction of NIH3T3 cells with pREV-TRE-hCD2t and pMSCV-tTA-IRES-EGFP, 23% of cells maintained in the absence of dox expressed both EGFP and CD2 (Figure 3.2A.iii). Interestingly, the mean fluorescence intensity (MFI) of CD2 expression was much higher in this $EGFP^+$ $CD2^+$ population than the MFI of the $CD2^+$ population observed following transduction with the CD2 construct alone. Therefore, tTA binding to the TRE elevated the expression levels of CD2. Following co-transduction, two distinct populations of CD2 expressing cells were present within the $EGFP^-$ $CD2^+$ population (Figure 3.2A.iii). One population of cells expressed low levels of CD2 with an MFI similar to that observed following transduction with the CD2 construct alone. The other population expressed much higher levels of CD2 with an MFI similar to that

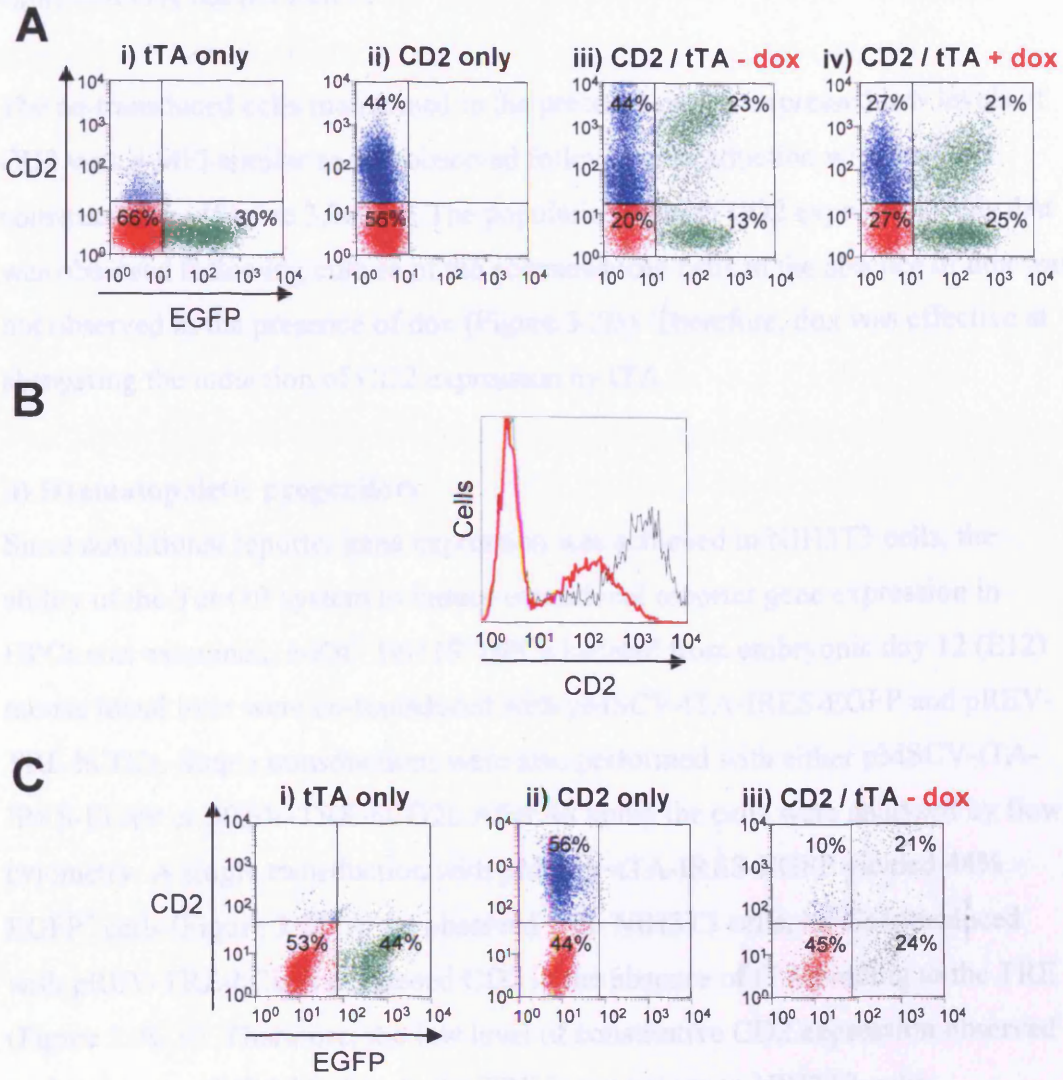

Figure 3.2 The Tet-Off system allows conditional reporter gene expression in both NIH3T3 cells and haematopoietic progenitor cells. A) NIH3T3 cells were transduced with either pREV-TRE-hCD2t (CD2 only) or pMSCV-tTA-IRES-EGFP (tTA only) or both of these constructs (CD2 / tTA). Co-transduced cells were maintained with or without dox for 5 days and then analysed by flow cytometry. **B)** The overlay plot shows the CD2 expression profile of NIH3T3 cells co-transduced with pREV-TRE-hCD2t and pMSCV-tTA-IRES-EGFP maintained in the presence (thick red line) or absence (grey line) of dox. The plots were gated on $EGFP^+$ cells. **C)** Haematopoietic progenitor cells (HPCs) were transduced with the same constructs as detailed in **A**. The cells were analysed 48 hours after transduction by flow cytometry.

observed in the $EGFP^+$ $CD2^+$ population in which tTA was inducing elevated levels of CD2 expression. Therefore, it is possible that despite the presence of the IRES in the pMSCV-tTA-IRES-EGFP construct, some cells transduced with this construct expressed tTA but not EGFP.

The co-transduced cells maintained in the presence of dox expressed low levels of CD2 with a MFI similar to that observed following transduction with the CD2 construct alone (Figure 3.2A.iv). The population of high CD2 expressing cells that was observed following culture of the co-transduced cells in the absence of dox was not observed in the presence of dox (Figure 3.2B). Therefore, dox was effective at abrogating the induction of CD2 expression by tTA.

ii) Haematopoietic progenitors

Since conditional reporter gene expression was achieved in NIH3T3 cells, the ability of the Tet-Off system to induce conditional reporter gene expression in HPCs was examined. c-Kit^+ $Ter119^-$ HPCs isolated from embryonic day 12 (E12) mouse foetal liver were co-transduced with pMSCV-tTA-IRES-EGFP and pREV-TRE-hCD2t. Single transductions were also performed with either pMSCV-tTA-IRES-EGFP or pREV-TRE-hCD2t. After 48 hours the cells were analysed by flow cytometry. A single transduction with pMSCV-tTA-IRES-EGFP yielded 44% $EGFP^+$ cells (Figure 3.2C.i). As observed with NIH3T3 cells, HPCs transduced with pREV-TRE-hCD2t expressed CD2 in the absence of tTA binding to the TRE (Figure 3.2C.ii). Therefore, the low level of constitutive CD2 expression observed in the absence of tTA binding to the TRE is not unique to NIH3T3 cells.

Following co-transduction of HPCs with pREV-TRE-hCD2t and pMSCV-tTA-IRES-EGFP, 20% of the cells co-expressed EGFP and CD2 (Figure 3.2C.iii). The MFI of CD2 expression in this $EGFP^+$ $CD2^+$ population was over an order of magnitude higher than that observed following transduction with the CD2 construct alone. Therefore, tTA was able to induce elevated levels of CD2 expression in HPCs. The ability of dox to abrogate this induction was not investigated in this experiment.

3.3 The Tet-suppressor abolishes low levels of constitutive tTA independent CD2 expression in NIH3T3 cells.

As described in section 3.2, a low level of constitutive CD2 expression was observed in the absence of tTA binding to the TRE. The hygromycin resistance gene, which is situated between the 5'LTR and the TRE, should prevent translational read-through of the messenger RNA (mRNA) expressed from the promoter elements of the 5'LTR. Either this was not the case here, or enhancer elements in the 5'LTR were acting on the minimal CMV promoter in the TRE resulting in low levels of constitutive CD2 expression. Strict regulatable reporter gene expression has been obtained when the TRE-reporter gene is in the anti-sense orientation with respect to the 5'LTR of the retroviral vector (Unsinger *et al.*, 2001). In order to test whether an anti-sense CD2 expression construct expressed lower levels of constitutive CD2, TRE-hCD2t was sub-cloned in the anti-sense orientation with respect to the 5'LTR of the pREV vector (Figure 2.1). This 'anti-sense' reporter expressed 2-fold lower levels of constitutive CD2 than the sense reporter in which TRE-hCD2t was in the sense orientation with respect to the 5'LTR (data not shown). The fact that constitutive CD2 expression was still observed using an anti-sense CD2 reporter implies that enhancer elements in the 5'LTR, which can act on the TRE regardless of its orientation, are responsible for the low levels of constitutive CD2 expression. Although the level of constitutive CD2 expression was substantially reduced using the anti-sense orientation construct, it was not completely abrogated. Therefore, the ability of the Tet suppressor (tTS) to abrogate the low level of constitutive tTA independent CD2 expression was investigated.

The Tet suppressor is a fusion of the TetR and the KRAB-AB silencing domain of the Kid-1 protein (Freundlieb *et al.*, 1999). tTS can bind to the TRE in the absence of dox. In doing so it may prevent enhancer elements in the 5'LTR from acting on the minimal CMV promoter in the TRE. In the presence of dox, tTS undergoes a conformational change such that it is inactivated and therefore no longer able to bind to the TRE (Figure 3.3). In order to test the ability of tTS to abrogate tTA independent constitutive CD2 expression, a bulk population of NIH3T3 cells that had been cultured for approximately 2 months following transduction with pREV-TRE-hCD2t were transduced with pMSCV-tTS-IRES-EGFP. The transduced cells

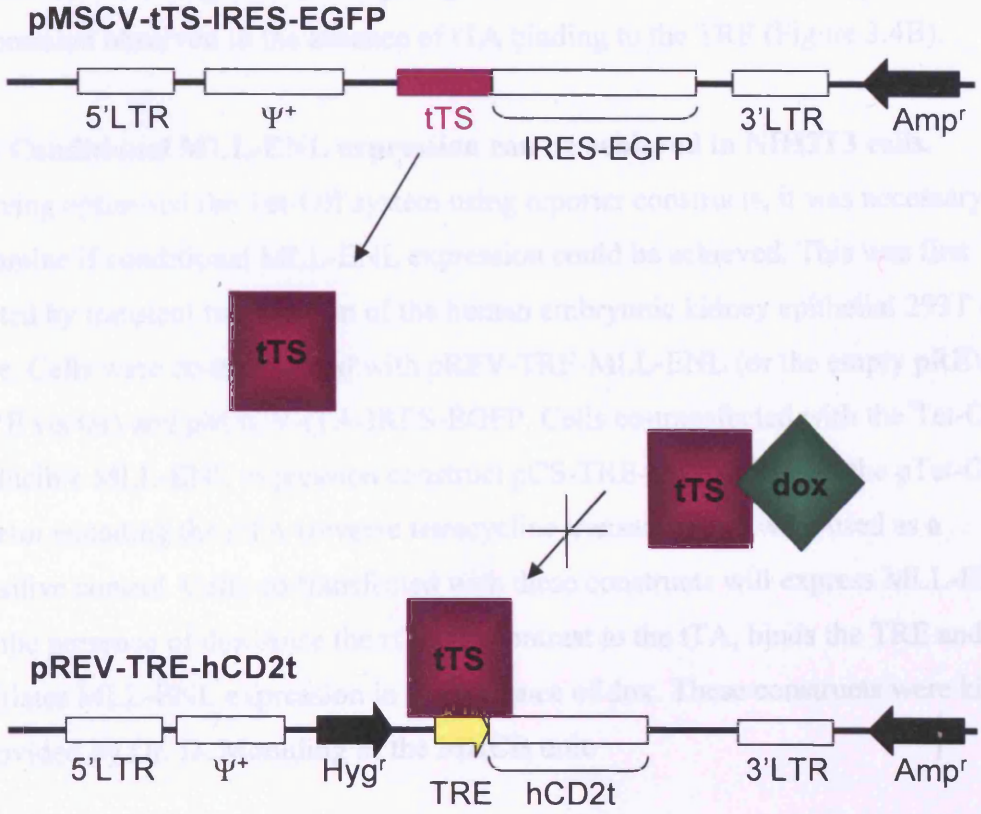

Figure 3.3 The function of the tetracycline suppressor. The tetracycline suppressor (tTS) binds to the same site in the TRE as tTA and may prevent enhancer elements in the 5'LTR from acting on the TRE, thereby preventing tTA independent CD2 expression. In the presence of dox, tTS is inactivated such that it cannot bind the TRE and can no longer suppress low levels of constitutive CD2 expression. LTR: long terminal repeat, ψ^+: viral packaging signal, tTS: tetracycline suppressor, IRES: internal ribosome entry site, EGFP: enhanced green fluorescent protein, Amp^r: ampicillin resistance gene, Hyg^r: hygromycin resistance gene, TRE: tetracycline response element, hCD2t: human CD2 tail-less.

were passaged after 4 days and maintained with or without dox for a further 5 days. The EGFP and CD2 expression profiles of the transduced cells were then analysed by flow cytometry. $EGFP^+$ cells, expressing tTS were almost completely CD2 negative (Figure 3.4A.ii). However, almost half of these cells were transduced with the CD2 expression construct since following the addition of dox, and tTS inactivation, they re-expressed CD2 (Figure 3.4A.iii). This data shows that tTS functioned as anticipated in abrogating the low levels of constitutive CD2 expression observed in the absence of tTA binding to the TRE (Figure 3.4B).

3.4 Conditional MLL-ENL expression can be achieved in NIH3T3 cells.

Having optimised the Tet-Off system using reporter constructs, it was necessary to examine if conditional MLL-ENL expression could be achieved. This was first tested by transient transfection of the human embryonic kidney epithelial 293T cell line. Cells were co-transfected with pREV-TRE-MLL-ENL (or the empty pREV-TRE vector) and pMSCV-tTA-IRES-EGFP. Cells co-transfected with the Tet-On inducible MLL-ENL expression construct pCS-TRE-MLL-ENL and the pTet-On vector encoding the rtTA (reverse tetracycline transactivator) were used as a positive control. Cells co-transfected with these constructs will express MLL-ENL in the presence of dox since the rtTA, in contrast to the tTA, binds the TRE and initiates MLL-ENL expression in the presence of dox. These constructs were kindly provided by Dr. D. Moulding of the MHCB unit.

Co-transfected cells were maintained in the presence or absence of dox for 24 hours and then lysed for Western blot analysis. Since MLL-ENL was myc-tagged at the 5' end in both the pREV-TRE-MLL-ENL and pCS-TRE-MLL-ENL constructs, the anti-myc antibody (clone 9E10) or the higher affinity anti-myc antibody (clone 9B11) were used to detect MLL-ENL protein expression. As expected a 220 kD band corresponding to full-length MLL-ENL was detected in pCS-TRE-MLL-ENL and rtTA co-transfected cells maintained in the presence of dox (the positive control) (Figure 3.5A). The full-length MLL-ENL protein was also detected in pREV-TRE-MLL-ENL and pMSCV-tTA-IRES-EGFP co-transfected cells maintained in the absence of dox (Figure 3.5A). However, it was not detected in co-

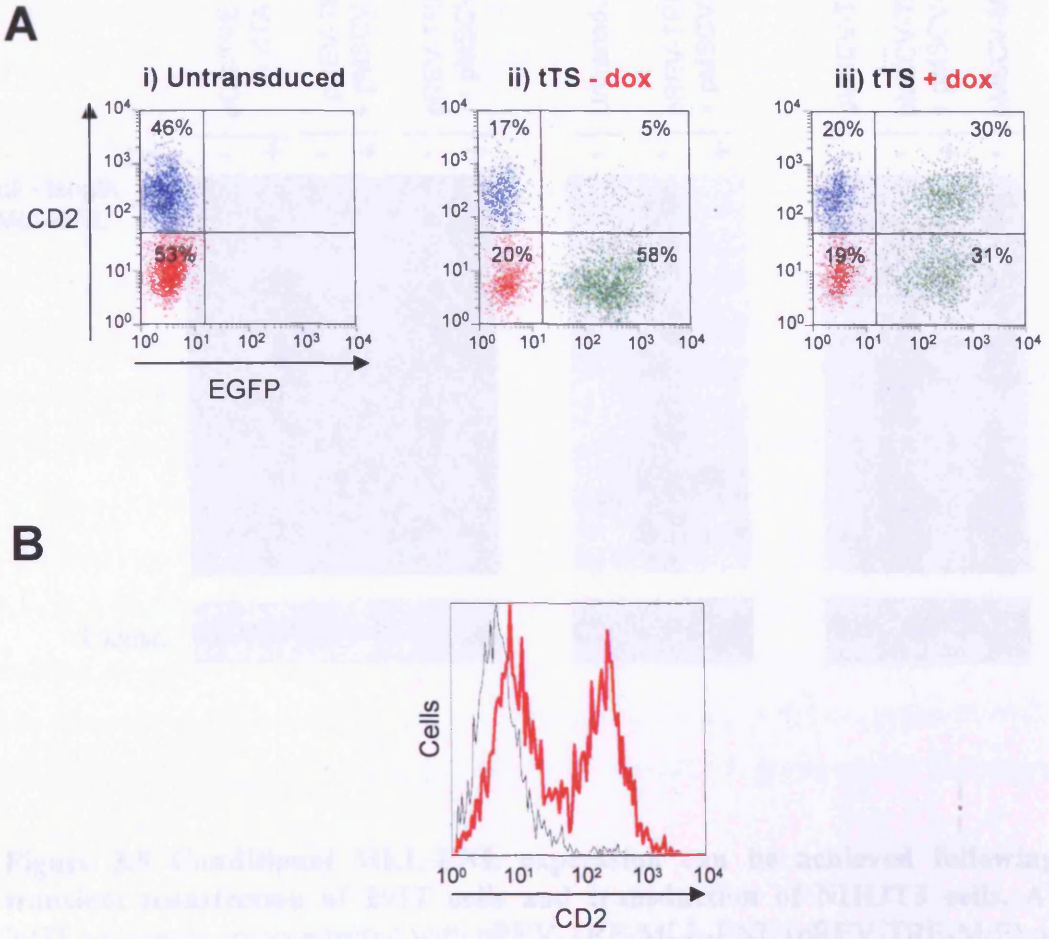

Figure 3.4 The tetracycline suppressor prevents tTA independent CD2 expression. A) A bulk culture of pREV-TRE-hCD2t transduced NIH3T3 cells that had been cultured for 2 months were transduced with pMSCV-tTS-IRES-EGFP. The co-transduced cells were maintained with or without dox for 5 days and then analysed by flow cytometry. **B)** The overlay plot shows the CD2 expression profile of NIH3T3 cells co-transduced with pREV-TRE-hCD2t and pMSCV-tTS-IRES-EGFP maintained in the presence (thick red line) or absence (grey line) of dox. Plots were gated on $EGFP^+$ cells.

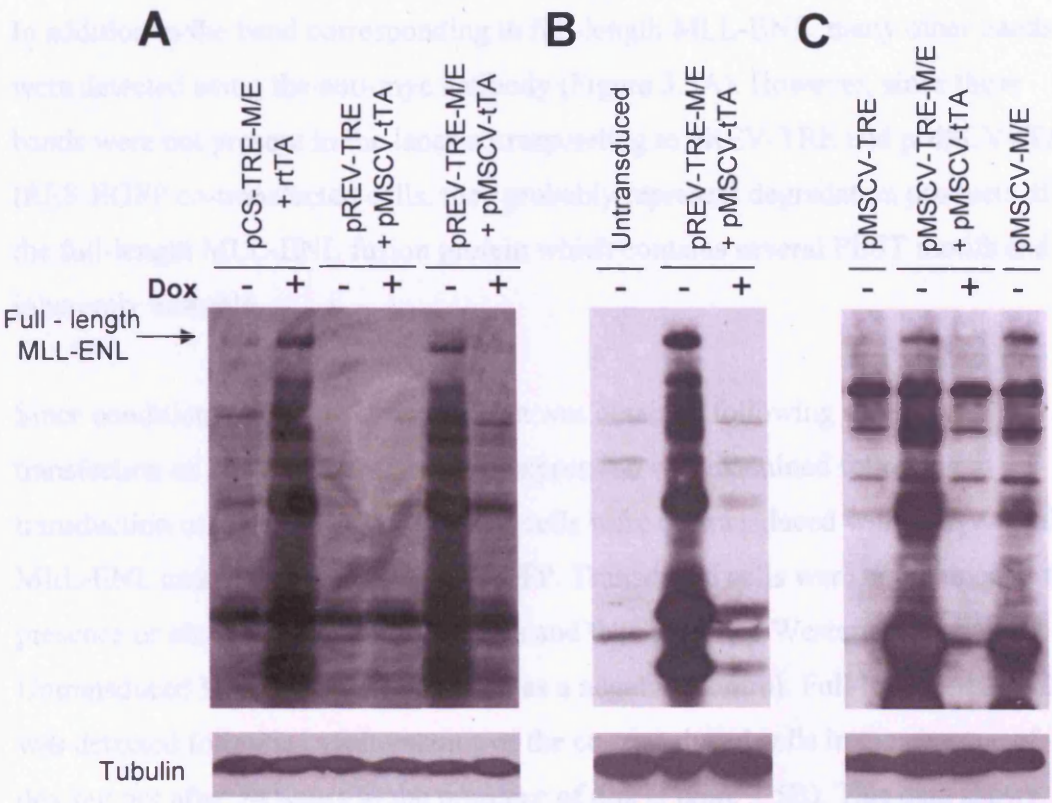

Figure 3.5 Conditional MLL-ENL expression can be achieved following transient transfection of 293T cells and transduction of NIH3T3 cells. A) 293T cells were co-transfected with pREV-TRE-MLL-ENL (pREV-TRE-M/E) or the empty vector (pREV-TRE) and pMSCV-tTA-IRES-EGFP (pMSCV-tTA). As a positive control, cells were co-transfected with the Tet-On expression constructs pCS-TRE-MLL-ENL (pCS-TRE-M/E) and the reverse tetracycline transactivator (rtTA). Co-transfected cells were maintained with or without dox for 24 hours and then lysed for Western blot analysis. MLL-ENL protein was detected with the anti-myc antibody (clone 9E10). **B)** NIH3T3 cells were co-transduced with pREV-TRE-M/E and pMSCV-tTA or **C)** pMSCV-TRE-M/E and pMSCV-tTA. Cells were also transduced with the constitutive MLL-ENL expression construct pMSCV-M/E. Untransduced NIH3T3 cells or empty vector (pMSCV-TRE) transduced cells served as a negative control. Co-transduced cells were maintained with or without dox for 24 hours and then lysed. The blot in **C** was probed with the high affinity anti-myc antibody (clone 9B11). Each blot was stripped and re-probed with an anti-tubulin antibody.

transfected cells maintained in the presence of dox. Therefore, regulatable MLL-ENL expression was obtained following transient transfection of 293T cells.

In addition to the band corresponding to full-length MLL-ENL, many other bands were detected using the anti-myc antibody (Figure 3.5A). However, since these bands were not present in the lanes corresponding to pREV-TRE and pMSCV-tTA-IRES-EGFP co-transfected cells, they probably represent degradation products of the full-length MLL-ENL fusion protein which contains several PEST motifs and is inherently unstable.

Since conditional MLL-ENL expression was obtained following transient transfection of 293T cells, MLL-ENL expression was examined following transduction of NIH3T3 cells. NIH3T3 cells were co-transduced with pREV-TRE-MLL-ENL and pMSCV-tTA-IRES-EGFP. Transduced cells were maintained in the presence or absence of dox for 24 hours and then lysed for Western blot analysis. Untransduced NIH3T3 cells were used as a negative control. Full-length MLL-ENL was detected following maintenance of the co-transduced cells in the absence of dox but not after 24 hours in the presence of dox (Figure 3.5B). This data shows that conditional MLL-ENL expression was achieved following integration of both the pMSCV-tTA-IRES-EGFP and pREV-TRE-MLL-ENL provirus into the genome of target cells.

Full-length MLL-ENL expression could not be detected in NIH3T3 cells transduced with a flag-tagged constitutive MLL-ENL expression construct using either anti-flag or anti-MLL antibodies. Therefore, a myc-tagged constitutive MLL-ENL expression construct was made so that the level of MLL-ENL protein expression could be directly compared in NIH3T3 cells transduced with either the constitutive or conditional MLL-ENL expression constructs. Full-length MLL-ENL was detected in cells transduced with the myc-tagged constitutive MLL-ENL expression construct (Figure 3.5C). Furthermore the amount of full-length MLL-ENL protein expressed was similar to that in cells co-transduced with pMSCV-tTA-IRES-EGFP and pMSCV-TRE-MLL-ENL (a new conditional MLL-ENL expression construct that produced a viral titre comparable to the constitutive construct). This new conditional MLL-ENL expression construct (Figure 2.2),

which is capable of producing higher titre retrovirus is discussed in more detail in chapter 4.

Discussion

Very few studies have employed two retroviral constructs to express a reporter that is strictly regulatable by tTA. Regulatable reporter gene expression has been obtained using a self-inactivating (SIN) retroviral vector containing a single autoregulatory cassette encoding both the tTA and the TRE-reporter gene (Hofmann *et al.*, 1996). SIN vectors contain deletions in the enhancer and promoter elements of the 3' LTR. These deletions are then transferred to the 5'LTR during reverse transcription, which results in transcriptional inactivation of the provirus. The inactivation of the enhancer and promoter elements in the 5'LTR is advantageous since it prevents their interference with the tTA regulated minimal CMV promoter within the TRE (Hofmann *et al.*, 1996). It was not possible to use a single autoregulatory expression construct to express MLL-ENL in a conditional manner since the MLL-ENL cDNA is 6.2 kb. A retrovirus encoding both MLL-ENL and tTA would not be packaged effectively since the size of the viral RNA would exceed the upper packaging constraint limit of 10 kb. Therefore, the efficacy of using two retroviral constructs to deliver the Tet-Off system to target cells was examined using reporter constructs.

A low level of constitutive reporter gene expression was observed in NIH3T3 cells and HPCs which were not transduced with the tTA expression construct. However, binding of tTA to the TRE did induce elevated reporter gene expression, which could be abrogated by dox. The low levels of constitutive, tTA independent, reporter gene expression posed a potential problem since low levels of constitutive MLL-ENL expression may be sufficient to immortalise HPCs. If this is the case dox would not turn off MLL-ENL expression, since expression would be independent of tTA binding to the TRE. Therefore, ways of shutting off the low levels of constitutive, tTA independent, reporter gene expression were investigated.

Strict regulatable reporter gene expression has been obtained in other studies when the TRE-reporter gene was in the anti-sense orientation with respect to the 5'LTR (Unsinger *et al.*, 2001). However, in this study low levels of constitutive CD2 expression were still observed using an anti-sense orientation TRE-hCD2t reporter. We hypothesised that the low levels of constitutive, tTA independent, reporter gene expression were caused by enhancer elements in the 5' LTR acting on the minimal

CMV promoter in the TRE. This is because enhancer elements are able to activate gene expression regardless of the orientation of the gene. If this was the case, it was possible that the Tet-suppressor, when bound to the TRE, might prevent enhancer elements in the 5'LTR from acting on the minimal CMV promoter of the TRE and therefore prevent tTA independent CD2 expression. This was found to be the case. Furthermore, in the presence of dox, tTS was no longer able to bind to the TRE and was not able to prevent tTA independent, CD2 expression. These experiments suggest that even in the event that a low level of constitutive MLL-ENL expression is sufficient to immortalise HPCs, transduction of the immortalised cell line with pMSCV-tTS should abrogate MLL-ENL expression and this may be regulated by dox.

The reporter constructs enabled us to optimise the delivery of the Tet-Off system to target cells and determine the efficacy with which the system allowed conditional gene expression in both NIH3T3 cells and HPCs. Having confirmed that the Tet-Off system allowed conditional reporter gene expression in these cells, it was important to determine whether we could achieve conditional MLL-ENL expression using this system. Conditional expression of the full-length MLL-ENL protein was achieved following transient co-transfection of 293T cells with the Tet-Off system. Importantly, conditional MLL-ENL expression was also achieved following co-transduction of NIH3T3 cells. Therefore, although the viral genome of the conditional MLL-ENL retroviral construct is large, the virus is packaged effectively and it can integrate into the genome of target cells. The finding that MLL-ENL expression is regulatable by tTA following integration of the virus into the genome of target cells, suggests that the promoters and enhancers of endogenous genes which flank the integration site do not interfere with the tTA regulated minimal CMV promoter within the TRE. This finding implies that we would be able to turn off MLL-ENL expression in cells immortalised with the conditional MLL-ENL expression construct.

Chapter 4 Analysis of the transforming capacity of MLL-ENL and MLL-AF4.

Previous studies have demonstrated that many MLL-fusion proteins including MLL-ENL and MLL-AF9 are leukaemogenic (Corral *et al.*, 1996; Lavau *et al.*, 1997). However, the leukaemogenic potential of the MLL-AF4 fusion protein, the product of the most prevalent chromosomal translocation in infant ALL, has not been assessed. Previous retroviral transduction studies have employed murine adult bone marrow as the source of haematopoietic progenitors for transduction with MLL fusion genes (Lavau *et al.*, 1997; Lavau *et al.*, 2000a; Lavau *et al.*, 2000b; DiMartino *et al.*, 2002; So and Cleary, 2002; So and Cleary, 2003). Since the translocation that generates the MLL-fusion gene is postulated to occur *in utero* (Eguchi *et al.*, 2003), HPCs isolated from the livers of E12 mouse foetuses may be a more appropriate target population for retroviral transduction. MLL-fusion genes may immortalise foetal HPCs and induce leukaemogenesis in mice which may more accurately mimic the disease in patients.

Purified populations of haematopoietic stem cells (HSCs), common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs) isolated from adult bone marrow are all susceptible to immortalisation by MLL-ENL (Cozzio *et al.*, 2003). The foetal equivalent of these three progenitor populations is present within the lin$^-$ c-Kit$^+$ population of E14 foetal liver (Traver *et al.*, 2001). We isolated the c-Kit$^+$ Ter-119$^-$ progenitor population from E12 foetal liver for retroviral transduction since E12 foetal liver contains 8-fold higher percentages of c-Kit$^+$ Ter-119$^-$ cells than E14 foetal liver (data not shown). Negative selection against the erythrocyte marker Ter-119 was performed since the majority of haematopoietic cells in the foetal liver are erythroid and MLL-ENL is unable to immortalise the megakaryocyte-erythroid progenitor (MEP) (Cozzio *et al.*, 2003).

4.1 MLL-ENL promotes the serial replating of foetal progenitors in methylcellulose.

In order to investigate whether MLL-ENL is capable of immortalising foetal HPCs, c-Kit$^+$ Ter-119$^-$ cells were transduced with MLL-ENL expressing retroviral constructs following overnight stimulation with stem cell factor (SCF), interleukin-6 (IL-6) and IL-3. Cells were co-transduced with the conditional MLL-ENL expression construct pREV-TRE-MLL-ENL (pREV-TRE-M/E) or a control empty vector (pREV-TRE) and the inducer pMSCV-tTA-IRES-EGFP (pMSCV-tTA). Cells were also transduced with the conditional MLL-ENL expression construct in the absence of pMSCV-tTA. This condition was included because a low level of constitutive CD2 expression was observed in the absence of tTA binding to the TRE (section 3.2), hence it is possible that low levels of constitutive MLL-ENL expression would be sufficient to immortalise HPCs. As a positive control, cells were transduced with the constitutive MLL-ENL expression construct pMSCV-MLL-ENL (pMSCV-M/E). Following two rounds of transduction the cells were cultured in methylcellulose (M3434) supplemented with GM-CSF and serial replating assays were performed. Immortalised cells possess the ability to self-renew and can therefore replate in methylcellulose indefinitely. However, non-immortalised cells progressively lose their self-renewal ability and terminally differentiate. Hygromycin was added to the first round of plating to select for cells transduced with pREV-TRE-M/E and G418 was used to select for cells transduced with pMSCV-M/E. After 6-10 days the colonies were counted, harvested and replated into a second round in the absence of selection.

Several of the primary colonies formed from pMSCV-M/E transduced cells were very large, round and compact (data not shown). In contrast, cells co-transduced with the empty pREV-TRE vector and pMSCV-tTA formed smaller granulocyte-macrophage (CFU-GM) colonies which were compact and of a non-uniform shape. Although a difference in colony morphology was observed, the total number of colonies formed and the total number of cells harvested was not significantly different between pMSCV-M/E and empty vector transduced cells in either the primary or secondary rounds of the assay (Figure 4.1A and B). However, more colonies were formed from cells transduced with pMSCV-M/E than the empty vector in the third round of replating (Figure 4.1A). The vast majority of the tertiary

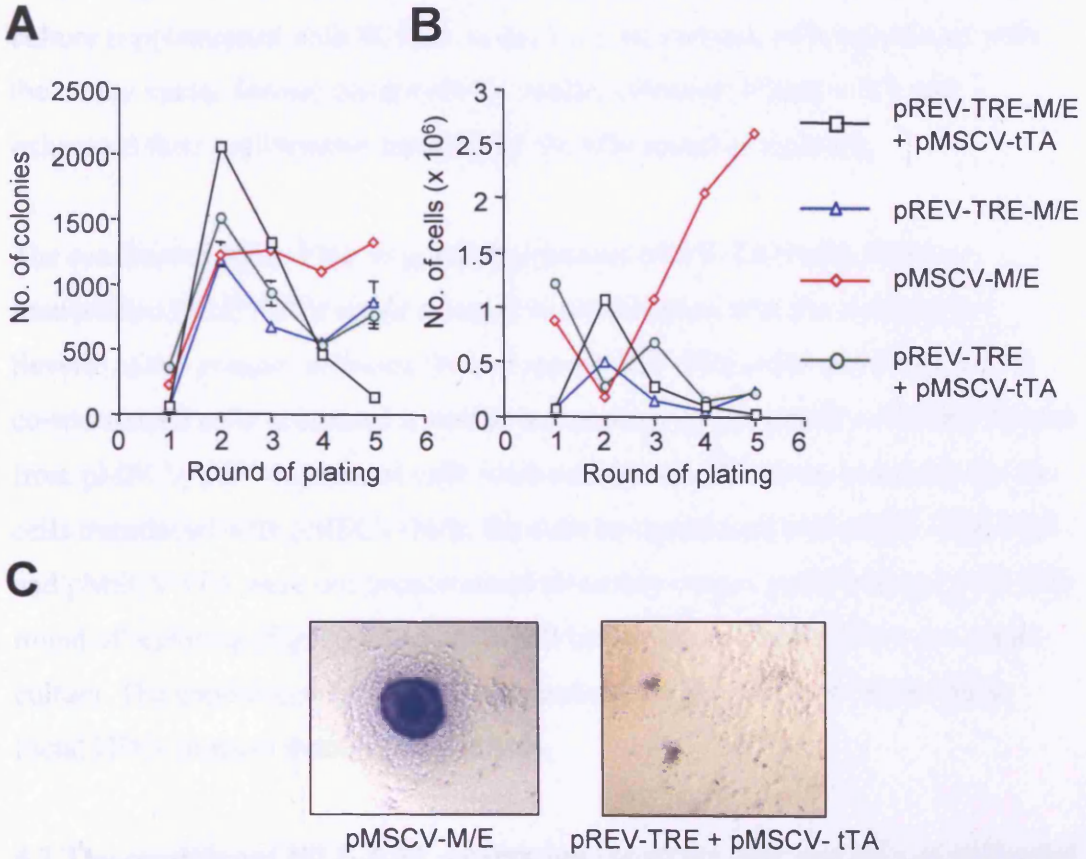

Figure 4.1 The constitutive MLL-ENL expression construct can immortalise foetal HPCs. E12 c-Kit$^+$ Ter-119$^-$ HPCs were co-transduced with the conditional MLL-ENL expression construct pREV-TRE-M/E (or the empty vector pREV-TRE) and the inducer pMSCV-tTA. Cells were also transduced with the constitutive MLL-ENL expression construct pMSCV-M/E. The transduced cells were cultured in methylcellulose under conditions that promoted myeloid development and serial replating assays were performed. Colonies were counted every 6-10 days and cells were harvested and replated into a subsequent round under the same conditions. The graphs show **A)** the number of colonies formed and **B)** the number of cells harvested (per 10^4 cells plated) in each round of the assay from cells transduced with pREV-TRE-M/E and pMSCV-tTA (black squares), pREV-TRE-M/E alone (blue triangles), pMSCV-M/E (red diamonds) and pREV-TRE and pMSCV-tTA (green circles). The mean and SD of duplicate cultures are shown in **A**. **C)** The morphology of colonies formed in the third round of the assay shown in **A** and **B**. Original magnification x 40.

pMSCV-M/E colonies were large and round. Some of them were surrounded by a halo of differentiating cells while others were more compact (Figure 4.1C). The pMSCV-M/E colonies replated consistently with the same frequency and morphology into subsequent rounds and a stable cell line was established in liquid culture supplemented with SCF, IL-6 and IL-3. In contrast, cells transduced with the empty vector formed progressively smaller colonies (Figure 4.1C) and exhausted their proliferative capacity by the fifth round of replating.

The conditional MLL-ENL expression construct pREV-TRE-M/E failed to immortalise foetal HPCs either alone or in combination with the inducer tTA. Several of the primary colonies formed from pREV-TRE-M/E and pMSCV-tTA co-transduced cells possessed a similar morphology to the primary colonies formed from pMSCV-M/E transduced cells (data not shown). However, in contrast to the cells transduced with pMSCV-M/E, the cells co-transduced with pREV-TRE-M/E and pMSCV-tTA were not immortalised since they ceased proliferating by the fifth round of replating (Figure 4.1A) and a cell line could not be established in liquid culture. The conditional MLL-ENL expression construct failed to immortalise foetal HPCs in more than 20 experiments.

4.2 The conditional MLL-ENL expression construct does not induce unlimited self-renewal of foetal progenitors in methylcellulose.

The inability of pREV-TRE-M/E to immortalise HPCs might be explained by its low viral titre. The pREV-TRE-M/E construct routinely produced viral titres of 2 x 10^5 ip/mL (infectious particles / mL), approximately 10-fold lower than that of pMSCV-M/E as measured by hygromycin or G418 resistance following transduction of NIH3T3 cells (section 2.5), data not shown. The way in which the co-transduction with pMSCV-tTA was performed reduced the titre of pREV-TRE-M/E further. The two viral supernatants were mixed in a 1:1 ratio, therefore the titre of each virus was effectively halved. In order to avoid this, the effect of performing a single transduction with pREV-TRE-M/E followed by a co-transduction with pREV-TRE-M/E and pMSCV-tTA, 24 hours later, was investigated. The effect of seeding twice the number of cells for transduction was also examined. This strategy yielded promising results. Although the total number of colonies formed was similar for all the constructs examined (Figure 4.2A), it is apparent that the cells co-

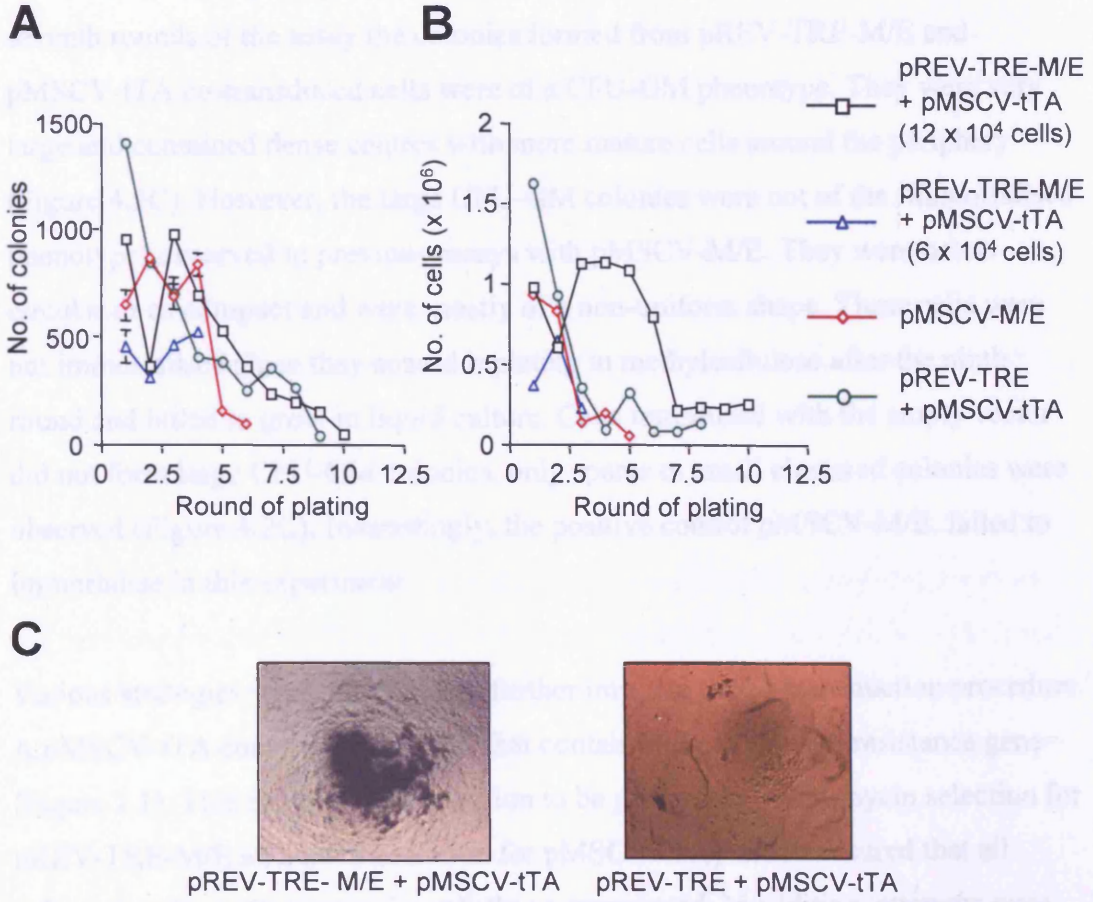

Figure 4.2 The conditional MLL-ENL expression construct causes a transient enhancement of self-renewal in foetal HPCs. E12 c-Kit$^+$ Ter-119$^-$ HPCs were co-transduced with the conditional MLL-ENL expression construct pREV-TRE-M/E (or the empty vector pREV-TRE) and the inducer pMSCV-tTA. Cells were also transduced with the constitutive MLL-ENL expression construct pMSCV-M/E. The transduced cells were cultured in methylcellulose under conditions that promoted myeloid development and serial replating assays were performed. The effect of plating twice the number of pREV-TRE-M/E and pMSCV-tTA transduced cells was investigated. The graphs show **A)** the number of colonies formed and **B)** the number of cells harvested (per 10^4 cells plated) in each round of the assay from cells transduced with pREV-TRE-M/E and pMSCV-tTA (12 x 10^4 cells) (black squares), pREV-TRE-M/E and pMSCV-tTA (6 x 10^4 cells) (blue triangles), pMSCV-M/E (red diamonds) and pREV-TRE and pMSCV-tTA (green circles). The mean and SD of duplicate cultures are shown in **A**. **C)** The morphology of colonies formed in the sixth round of the assay shown in **A** and **B**. Original magnification x 40.

transduced with pREV-TRE-M/E and pMSCV-tTA formed much larger colonies, as shown by the larger number of cells harvested from each plate (Figure 4.2B). This effect was only observed when more cells were plated. Between the third and seventh rounds of the assay the colonies formed from pREV-TRE-M/E and pMSCV-tTA co-transduced cells were of a CFU-GM phenotype. They were very large and contained dense centres with more mature cells around the periphery (Figure 4.2C). However, the large CFU-GM colonies were not of the 'immortalised phenotype' observed in previous assays with pMSCV-M/E. They were not as circular or as compact and were mostly of a non-uniform shape. These cells were not immortalised since they ceased replating in methylcellulose after the ninth round and failed to grow in liquid culture. Cells transduced with the empty vector did not form large CFU-GM colonies, only sparse or small clustered colonies were observed (Figure 4.2C). Interestingly, the positive control pMSCV-M/E, failed to immortalise in this experiment.

Various strategies were employed to further improve the co-transduction procedure. A pMSCV-tTA construct was made that contained the neomycin resistance gene (Figure 2.1). This enabled dual selection to be performed (hygromycin selection for pREV-TRE-M/E and G418 selection for pMSCV-tTA) which ensured that all colony-forming cells were successfully co-transduced. In addition, attempts were made to increase the viral titre by concentrating the viral supernatants by centrifugation (section 2.7). This technique did not work for pREV-TRE-M/E. However, it was possible to concentrate the pMSCV-tTA virus. This was beneficial since co-transduction could then be performed without diluting the pREV-TRE-M/E virus (the concentrated pMSCV-tTA viral pellet was resuspended in neat pREV-TRE-M/E virus). Despite these improvements to the co-transduction procedure, it was not possible to generate a conditional immortalised cell line from foetal liver. Therefore, a new conditional MLL-ENL expression construct that was capable of generating higher titre virus was made by sub-cloning TRE-MLL-ENL downstream of PGK-neo in pMSCV-neo (Figure 2.2). pMSCV-TRE-M/E routinely gave viral titres of 2×10^6 ip / mL which was comparable to that of the constitutive MLL-ENL construct pMSCV-M/E. Unfortunately co-transduction of foetal HPCs with the new conditional MLL-ENL construct and pMSCV-tTA did not yield an immortalised cell line.

4.3 MLL-ENL immortalised cells are capable of long-term growth *in vitro*.

Two immortalised cell lines (FL-ME1 and FL-ME2) were generated from HPCs transduced with the constitutive MLL-ENL expression construct pMSCV-M/E in two independent experiments. The cell lines were generated by placing cells harvested from the fourth round of the methylcellulose replating assay in liquid culture supplemented with SCF, IL-6 and IL-3. Both FL-ME1 and FL-ME2 proliferated rapidly while cells co-transduced with the empty vector pREV-TRE and pMSCV-tTA grew more slowly and stopped proliferating after two weeks in liquid culture (Figure 4.3A). The growth kinetics of cells transduced with the conditional MLL-ENL expression construct either alone or in combination with tTA mirrored that of the empty vector transduced cells (data not shown).

The presence of the intact 6 kb MLL-ENL provirus was confirmed in both FL-ME1 and FL-ME2 by Southern blotting using an N-terminal MLL cDNA fragment as a probe (Figure 4.3B). A 12 kb band corresponding to an endogenous MLL fragment was also detected using this probe as expected. MLL-ENL protein expression could not be detected in FL-ME1 or FL-ME2 by Western blot analysis using either anti-FLAG or anti-MLL (clone 4.1) antibodies. Therefore, MLL-ENL transcript expression was examined by RT-PCR analysis using primers that flank the MLL-ENL breakpoint. The expected 846 bp product was amplified from total RNA isolated from FL-ME2 but not from FL-ME1 (Figure 4.3C). It has been previously reported that the level of MLL-ENL transcript expression in immortalised cell lines is so low that it can only be detected by performing RT-PCR on poly (A) RNA (Lavau *et al.*, 1997). Therefore, it may be necessary to isolate poly (A) RNA from FL-ME1 and repeat this analysis. It may also be necessary to use primers which generate a shorter product than 846 bp.

4.4 Characterisation of MLL-ENL immortalised cell lines.

The morphology of FL-ME1 and FL-ME2 was examined by cytospin preparation followed by May Grunwald Giemsa (MGG) staining. Both lines were composed predominantly of myeloblasts. However, some cells were at a more advanced stage of myeloid differentiation since they possessed a more segmented nucleus (Figure 4.4A). Analysis of cell surface antigen expression by flow cytometry confirmed that these cell lines were myeloid (Figure 4.4B). Both lines expressed high levels of the

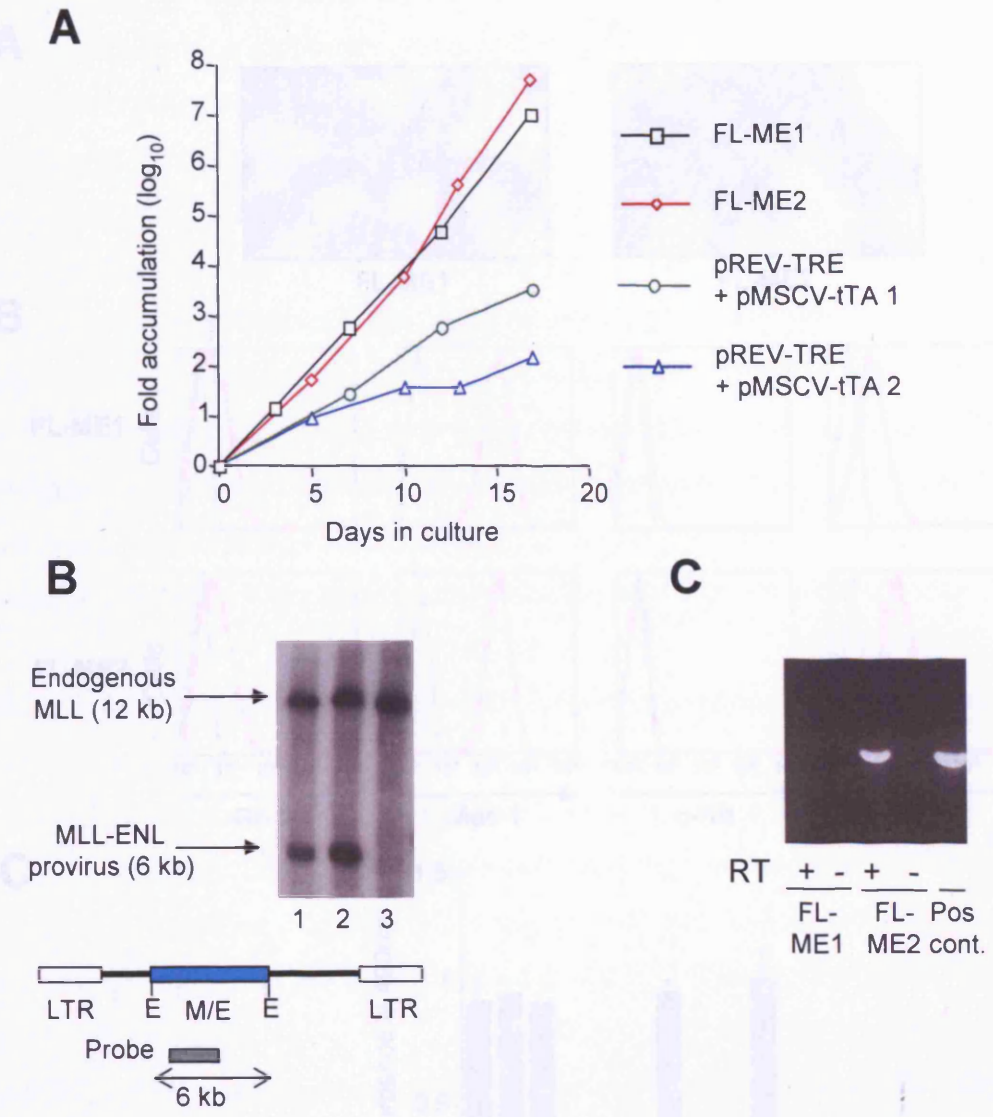

Figure 4.3 Stable cell lines can be established in liquid culture from MLL-ENL transduced foetal HPCs. Cells harvested from the fourth round of two independent methylcellulose assays were placed in liquid culture supplemented with SCF, IL-3 and IL-6. **A)** The graph shows the log of the fold accumulation in cell number of FL-ME1 (black squares) and FL-ME2 (red diamonds) which were derived from pMSCV-M/E transduced cells. The fold accumulation of cells transduced with the empty vector pREV-TRE and pMSCV-tTA (green circles and blue triangles) is also shown. **B)** Southern blot analysis of genomic DNA isolated from FL-ME1 (lane 1) and FL-ME2 (lane 2) showing integration of the MLL-ENL provirus. Untransduced E12 c-Kit$^+$ Ter-119$^-$ cells were used as a negative control (lane 3). The indicated N-terminal MLL cDNA fragment probe was used to detect the 6 kb proviral band following *EcoRI* digestion of genomic DNA. The probe detects a fragment of the endogenous MLL gene (top arrow) and the MLL-ENL provirus (bottom arrow). E indicates *EcoRI.* **C)** Reverse-transcription (RT)-PCR analysis of total RNA isolated from the cell lines demonstrating expression of the MLL-ENL transcript. The pCSARQ2 template was used as a positive control (pos cont). Negative control reactions were performed in the absence of reverse transcriptase (RT).

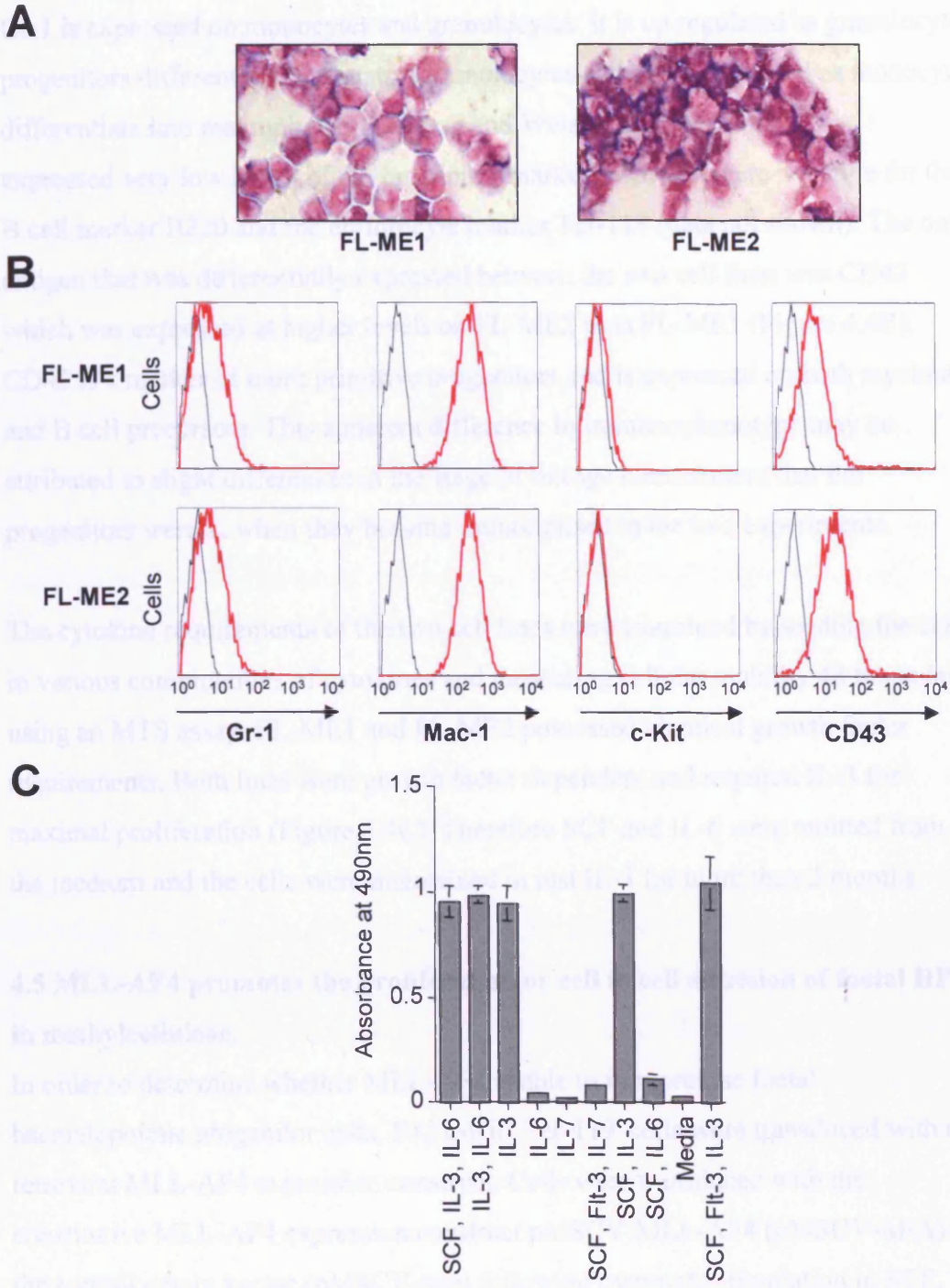

Figure 4.4 Characterisation of MLL-ENL cell lines derived from foetal HPCs. A) The cellular morphology of the cell lines was assessed by cytospin preparation followed by May Grunwald Giemsa (MGG) staining. Original magnification x 400. **B)** Flow cytometric analysis of cell surface antigen expression by the cell lines. Thick red lines represent the expression profile of the indicated antigen and thin black lines represent the appropriate isotype control. **C)** Growth factor requirements of FL-ME1. The graph shows the absorbance from MTS assays performed following culture with the indicated cytokines for 48 hours. Identical results were obtained for FL-ME2. The mean and SD of triplicate absorbance readings are shown.

macrophage marker Mac-1 and intermediate levels of the granulocyte marker Gr-1. Gr-1 is expressed on monocytes and granulocytes. It is up regulated as granulocyte progenitors differentiate into mature granulocytes and down regulated as monocytes differentiate into macrophages (Lagasse and Weissman, 1996). Both lines expressed very low levels of the progenitor marker c-Kit and were negative for the B cell marker B220 and the erythrocyte marker Ter-119 (data not shown). The only antigen that was differentially expressed between the two cell lines was CD43 which was expressed at higher levels on FL-ME2 than FL-ME1 (Figure 4.4B). CD43 is a marker of more primitive progenitors and is expressed on both myeloid and B cell precursors. This apparent difference in immunophenotype may be attributed to slight differences in the stage of lineage commitment that the progenitors were at when they became immortalised in the two experiments.

The cytokine requirements of the two cell lines were examined by seeding the cells in various combinations of cytokines and measuring cellular viability 48 hours later using an MTS assay. FL-ME1 and FL-ME2 possessed identical growth factor requirements. Both lines were growth factor dependent and required IL-3 for maximal proliferation (Figure 4.4C). Therefore SCF and IL-6 were omitted from the medium and the cells were maintained in just IL-3 for more than 3 months.

4.5 MLL-AF4 promotes the proliferation or cell to cell adhesion of foetal HPCs in methylcellulose.

In order to determine whether MLL-AF4 is able to immortalise foetal haematopoietic progenitor cells, E12 c-Kit$^+$ Ter-119$^-$ cells were transduced with a retroviral MLL-AF4 expression construct. Cells were transduced with the constitutive MLL-AF4 expression construct pMSCV-MLL-AF4 (pMSCV-M/A) or the control empty vector (pMSCV-neo) following overnight stimulation in SCF, IL-6 and IL-3. Following two rounds of transduction the cells were cultured in methylcellulose that supported B cell development (M3231 supplemented with SCF, IL-7 and *fms*-like tyrosine kinase-3 ligand [Flt-3 ligand]). These conditions were used because the MLL-AF4 translocation is predominantly associated with lymphoid leukaemias and a previous study used these conditions to generate MLL-ENL immortalised B cell lines (Zeisig *et al.*, 2003a). In some experiments, the

transduced cells were also plated in methylcellulose that supported myeloid development (M3434 supplemented with GM-CSF).

Flow cytometric analysis of cells harvested from the primary methylcellulose plating demonstrated that pMSCV-M/A and pMSCV-neo transduced cells failed to generate B cells (data not shown). This may have been because IL-3 can inhibit B cell commitment (Hirayama *et al*, 1994). Therefore, in subsequent experiments cells were transduced in the presence of SCF, IL-7 and Flt-3 ligand and then plated in M3231 methylcellulose supplemented with the same cytokines. Transduced cells were not selected since the PGK promoter and the neomycin gene were removed from the pMSCV-M/A construct (Figure 2.2). The neomycin resistance gene and its promoter were removed since the MLL-AF4 cDNA is 7.6 kb. A retrovirus encoding both the MLL-AF4 and the neomycin gene would not be packaged effectively since the size of the viral RNA would exceed the upper packaging constraint limit of 10 kb.

Although there was no difference in the number or the morphology of colonies formed from pMSCV-M/A and pMSCV-neo transduced cells grown in myeloid conditions (data not shown), a difference in colony morphology was observed when the transduced cells were grown in B cell conditions. More primary B cell colonies were formed from cells transduced with pMSCV-M/A than pMSCV-neo (Figure 4.5A). However, it is apparent from the number of cells harvested that the primary and secondary colonies formed from pMSCV-neo transduced cells were much larger than the colonies formed from pMSCV-M/A transduced cells (Figure 4.5B). The primary and secondary colonies formed from pMSCV-neo transduced cells were very large, dense and non-uniform in shape. In contrast, the colonies formed from pMSCV-M/A transduced cells were generally smaller, more circular and possessed a more compact centre (data not shown). However, by the third round of replating, many of the colonies formed from pMSCV-M/A transduced cells were much larger than the colonies formed from pMSCV-neo transduced cells (Figure 4.5C). The former were large, round and compact whereas the latter were smaller and more diffuse. Although the colonies formed from pMSCV-M/A and pMSCV-neo transduced cells were vastly different in terms of their size, the total number of cells harvested was not significantly different (Figure 4.5B) and their

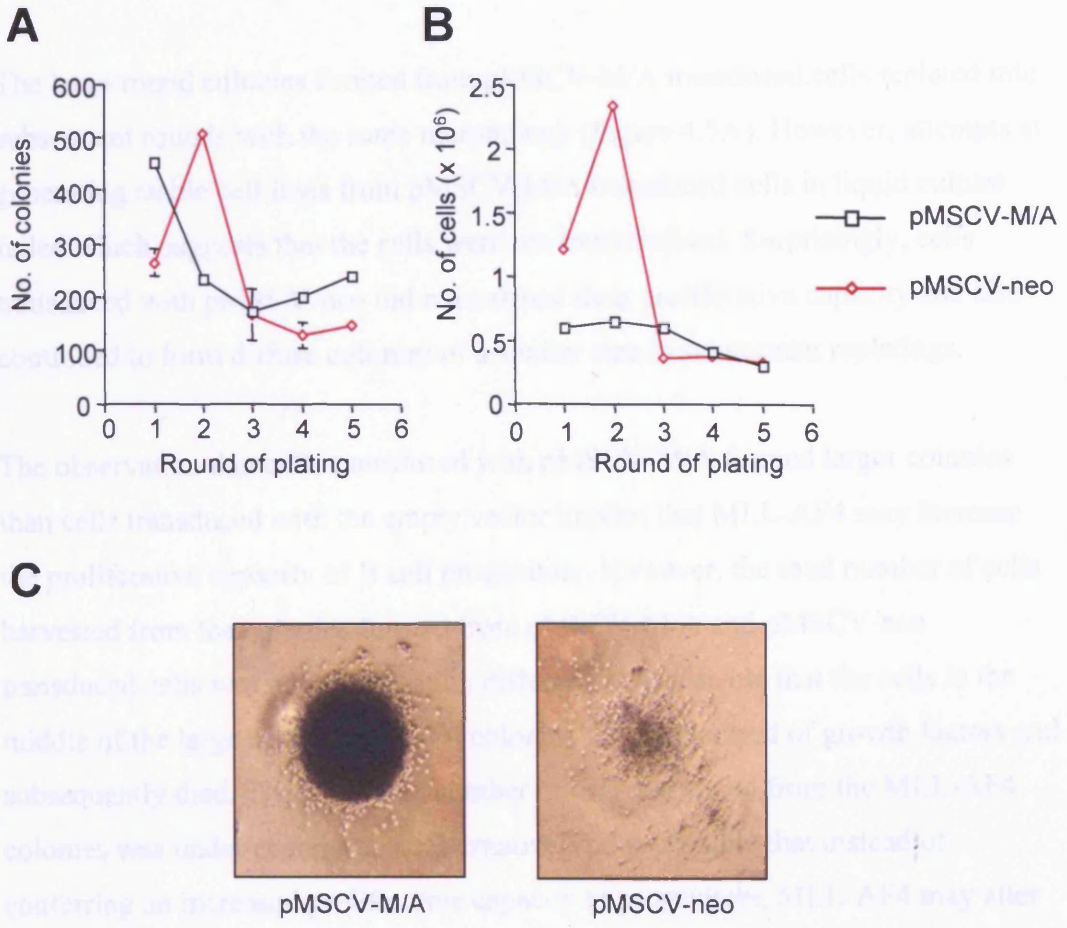

Figure 4.5 MLL-AF4 promotes the proliferation or cell to cell adhesion of foetal HPCs. E12 c-Kit$^+$ Ter-119$^-$ HPCs were transduced with the constitutive MLL-AF4 expression construct pMSCV-M/A or the empty vector pMSCV-neo. Transduced cells were cultured in methylcellulose under conditions that promoted B cell development and serial replating assays were performed. The graphs show **A)** the number of colonies formed and **B)** the number of cells harvested in each round of the assay from cells transduced with pMSCV-M/A (black squares) and pMSCV-neo (red diamonds) per 10^4 cells plated. The mean and SD of duplicate cultures are shown in **A**. **C)** The morphology of colonies formed in the third round of the assay shown in **A** and **B**. Original magnification x 40.

immunophenotype was identical (Figure 4.6). Both pMSCV-M/A and pMSCV-neo transduced cells expressed high levels of the B cell markers B220, CD19 and BP-1. They also expressed the progenitor marker c-Kit and were negative for the myeloid marker Mac-1 (Figure 4.6). This cell surface antigen expression profile is consistent with that of a pre-B cell phenotype (Hardy and Hayakawa, 2001).

The large round colonies formed from pMSCV-M/A transduced cells replated into subsequent rounds with the same morphology (Figure 4.5A). However, attempts at generating stable cell lines from pMSCV-M/A transduced cells in liquid culture failed which suggests that the cells were not immortalised. Surprisingly, cells transduced with pMSCV-neo did not exhaust their proliferative capacity and also continued to form diffuse colonies of a similar size in subsequent replatings.

The observation that cells transduced with pMSCV-M/A formed larger colonies than cells transduced with the empty vector implies that MLL-AF4 may increase the proliferative capacity of B cell progenitors. However, the total number of cells harvested from the colonies formed from pMSCV-M/A and pMSCV-neo transduced cells was not significantly different. It is possible that the cells in the middle of the large dense MLL-AF4 colonies were exhausted of growth factors and subsequently died. Therefore, the number of cells harvested from the MLL-AF4 colonies was under-represented. Alternatively, it is possible that instead of conferring an increased proliferative capacity to progenitors, MLL-AF4 may alter the expression of cell surface adhesion molecules such that the cells aggregate to form more compact, larger colonies. This result was only observed in 1 out of 4 experiments hence further experiments are required to reproduce this data and validate this hypothesis.

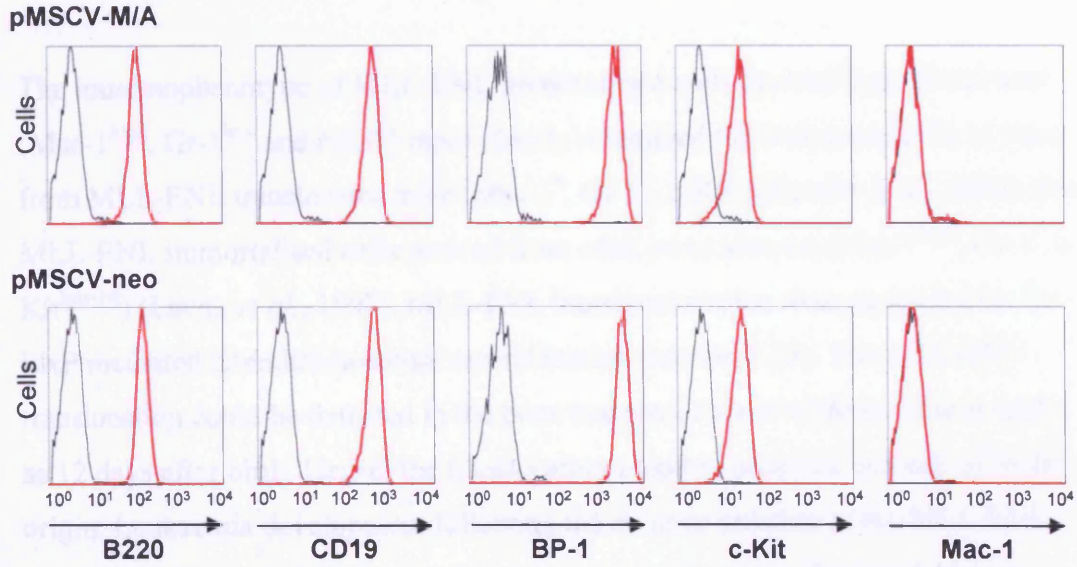

Figure 4.6 MLL-AF4 does not alter the differentiation of foetal HPCs. Cells transduced with the MLL-AF4 expression construct (pMSCV-M/A) and the empty vector (pMSCV-neo) were harvested from the fourth round of the methylcellulose serial replating assay and their cell surface antigen expression was analysed by flow cytometry. Thick red lines represent the expression profile of the indicated antigen and thin black lines represent the appropriate isotype control.

Discussion

The constitutive MLL-ENL expression construct pMSCV-M/E was able to immortalise c-Kit$^+$ Ter-119$^-$ foetal HPCs. The immortalised cells were capable of growth in liquid culture and two IL-3-dependent myeloid cell lines (FL-ME1 and FL-ME2) were established. Surprisingly, cells co-transduced with the empty-vector pREV-TRE and the inducer pMSCV-tTA did not exhaust their proliferative capacity until the fifth round of replating. Therefore, foetal HPCs have a greater capacity for self-renewal than that previously reported for HPCs isolated from bone marrow (Lavau *et al.*, 1997). This is probably due to the increased percentage of HSCs in foetal liver compared to bone marrow (Morrison *et al.*, 1995).

The immunophenotype of MLL-ENL immortalised cells derived from foetal liver (Mac-1^{high}, Gr-1^{low} and c-Kit$^-$) more closely resembled the leukaemic cells isolated from MLL-ENL translocator mice (Mac-1$^+$, Gr-1$^+$, c-Kit$^-$) (Forster *et al.*, 2003) than MLL-ENL immortalised cells derived from adult bone marrow (Mac-1^{high}, Gr-1$^-$, c-Kit$^{low-int}$) (Lavau *et al.*, 1997). MLL-ENL translocator mice were generated by Cre-loxP mediated interchromosomal recombination (section 1.13). The MLL-ENL translocation could be detected in the bone marrow of some of these mice as early as 12 days after birth. Hence, the translocation possibly occurred in a cell of foetal origin. Leukaemia development following the *de novo* creation of the MLL-ENL translocation accurately mimics what occurs in patients since the translocation occurs specifically in haematopoietic cells and is likely to be a low frequency event. The fact that MLL-ENL cell lines derived from foetal HPCs are more similar to the leukaemic translocator mouse cells than MLL-ENL cell lines derived from adult HPCs suggests that retroviral transduction of foetal HPCs, rather than bone marrow HPCs, may more closely mimic the disease in patients.

The conditional MLL-ENL expression construct pREV-TRE-M/E consistently failed to immortalise foetal HPCs either alone, or in combination with the inducer pMSCV-tTA. A transient increase in self-renewal was observed in one experiment. However, the cells did not replate indefinitely in methylcellulose and they failed to grow in liquid culture. It is likely that successful co-transduction of the target cell capable of immortalisation with two retroviral constructs was a rare event. Various strategies were employed to increase the co-transduction efficiency. These included

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the generation of a pMSCV-tTA expression construct with a selectable marker to enable dual selection, concentration of the pMSCV-tTA virus, and generation of a new conditional MLL-ENL expression construct which produced higher titres of retrovirus. However, all of these approaches were unsuccessful in generating a conditional immortalised cell line derived from foetal HPCs. The fact that the constitutive MLL-ENL construct pMSCV-M/E yielded immortalised cell lines in only 2 out of 6 experiments suggested that the target cell susceptible to immortalisation by MLL-ENL was not present in sufficiently high numbers within the c-Kit$^+$ Ter-119$^-$ population to give reproducible results. c-Kit is highly expressed on HSCs, CMPs and GMPs in E14 foetal liver (Traver *et al.*, 2001). However, c-Kit is also expressed on common lymphoid progenitors (CLPs) and megakaryocyte-erythroid progenitors (MEPs) (Traver *et al.*, 2001). MLL-ENL is not able to immortalise the adult MEP or CLP (Cozzio *et al.*, 2003), therefore the MEPs and CLPs present within the c-Kit$^+$ Ter-119$^-$ population of E12 foetal liver may be diluting the populations of progenitors that are susceptible to immortalisation by MLL-ENL. It is possible that a population of foetal HPCs further enriched in HSCs may give more reproducible results. This is described in more detail in chapter 5.

Preliminary experiments suggest that MLL-AF4 promotes the proliferation or cell to cell adhesion of foetal B cell progenitors. HPCs transduced with an MLL-AF4 expression construct formed much larger colonies than cells transduced with an empty vector. However, the total number of cells harvested was not significantly different and both the MLL-AF4 and empty vector transduced cells exhibited an identical pre-B cell immunophenotype. The pre-B cell phenotype of the MLL-AF4 transduced cells was surprising since leukaemic blasts isolated from patients with MLL-AF4 translocations usually have a pro-B cell phenotype (Eguchi *et al.*, 2003). The pro-B cell leukaemic blasts also frequently express myeloid antigens. MLL-AF4 transduced cells lacked expression of the myeloid antigen Mac-1 and did not generate stable cell lines in liquid culture. Further experiments need to be performed in order to determine if these results are reproducible. It will be important to include a positive control for immortalisation given the inconsistency in generating myeloid MLL-ENL immortalised cell lines from c-Kit$^+$ Ter-119$^-$ HPCs. As with MLL-ENL, it may be important to obtain a purer population of stem

cells for transduction. The frequent co-expression of myeloid antigens by pro-B cell leukaemic blasts isolated from patients harbouring 11q23 translocations suggests that a stem cell or a cell with bipotent B cell / monocytic differentiation capacity is the target for transformation.

Since the t(4;11) translocation is one of the most prevalent translocations in infant ALL and the majority of MLL fusion proteins studied to date are oncogenic, it would be very surprising if MLL-AF4 lacked oncogenic potential. However, it is possible that MLL-AF4 is not oncogenic by itself and that secondary genetic events are required for immortalisation. MLL-AF4 may confer a pre-leukaemic phenotype to B cell progenitors by enhancing their proliferative capacity and creating an expanded pool of B cells in which secondary mutations can occur. Recent studies suggest that Flt-3 gene mutations are secondary genetic events which collaborate with MLL fusion proteins to induce leukaemia. Point mutations in the activation loop of the tyrosine kinase domain of Flt-3 were found in 18% of infant ALL patients with *MLL*-rearrangements (Taketani *et al.*, 2004). Furthermore, over-expression of a constitutively active mutant Flt-3 receptor bearing an internal tandem duplication (Flt3-ITD) co-operated with MLL-ENL and MLL-SEPT6 to accelerate leukaemogenesis (Ono *et al.*, 2005). It will be interesting to examine whether a constitutively active Flt-3 receptor can co-operate with MLL-AF4 to immortalise HPCs.

An alternative hypothesis is that the reciprocal translocation product AF4-MLL confers oncogenic activity. This hypothesis is supported by the fact that AF4-MLL can transform murine embryonic fibroblasts yet MLL-AF4 cannot (Bursen *et al.*, 2004). The hypothesis is controversial since the AF4-MLL fusion gene is not expressed in all t(4;11) patients (Downing *et al.*, 1994). One theory is that AF4-MLL may operate by way of a "hit and run mechanism" such that AF4-MLL is expressed at disease onset but expression is lost as subsequent mutations occur (Bursen *et al.*, 2004). It will be interesting to examine whether AF4-MLL is able to immortalise HPCs and if MLL-AF4 can co-operate with AF4-MLL to induce leukaemia.

Another possibility is that MLL-AF4 is oncogenic in human HPCs but not in murine HPCs. This may be due to differences in the transcriptional regulation of haematopoiesis between mice and humans. Interestingly, it has recently been reported that a dominant negative mutant CEBPα protein induces a differentiation block in human but not murine myeloid progenitors (Schwieger *et al.*, 2004). Differences in the transcriptional regulation of the CEBPα gene, or CEBPα target genes, between mouse and human progenitors may explain these results. There is evidence to suggest that some genes might be differentially regulated in human and murine haematopoietic cells. For example, the CD34 gene is highly expressed by the vast majority of human HSCs (Baum *et al.*, 1992), yet murine HSCs do not express significant levels of this gene (Osawa *et al.*, 1996). Instead, CD34 is up-regulated at a slightly later stage in murine haematopoiesis (Okuno *et al.*, 2002). It is possible therefore, that murine and human HSCs possess slightly different transcriptional programmes and that the murine HSC transcriptional programme may not facilitate immortalisation by MLL-AF4. There may also be differences in the transcriptional programme of murine and human B cell progenitors. This is because the signalling pathways governing B cell differentiation are more complex in humans than those in mice. Mice are dependent on IL-7 for B cell development since IL-7 receptor deficient mice lack B cells (Miller *et al.*, 2002). However, other signalling pathways exist in humans that promote B cell development since individuals which lack expression of the IL-7 receptor possess normal numbers of B cells (Puel *et al.*, 1998). The differences in cytokine requirements of human and murine B cell progenitors may ultimately result in different transcriptional programmes in these cells which may affect their susceptibility to immortalisation by MLL-AF4.

Chapter 5 Establishment of conditional MLL-ENL immortalised cell lines.

Retroviral transduction of E12 c-Kit$^+$ Ter-119$^-$ HPCs with the constitutive MLL-ENL expression construct pMSCV-M/E yielded immortalised cell lines in 2 out of 6 experiments. However co-transduction of this HPC population with the conditional MLL-ENL expression construct pREV-TRE-M/E or pMSCV-TRE-M/E (which generated higher titre retrovirus) and the inducer pMSCV-tTA failed to immortalise. We hypothesised that the c-Kit$^+$ Ter-119$^-$ population may not contain the target cell, which is susceptible to immortalisation by MLL-ENL, at a high frequency. Therefore, a population of HPCs further enriched in HSCs was isolated. Since all long term reconstituting foetal HSCs are Mac-1$^+$, c-Kit$^+$ and Sca-1$^+$ (Morrison *et al.*, 1995), the Sca-1$^+$ c-Kit$^+$ HPC population from E12 foetal liver was purified by fluorescence activated cell sorting (FACS). The ability of MLL-ENL to immortalise this enriched population of foetal HSCs was studied and compared to the ability of MLL-ENL to immortalise unsorted adult HPCs isolated from bone marrow of 5-fluorouracil (5-FU) treated mice.

5.1 The conditional MLL-ENL expression construct can immortalise HPCs isolated from bone marrow but not foetal liver.

The ability of MLL-ENL to immortalise E12 Sca-1$^+$ c-Kit$^+$ foetal HPCs or unsorted adult HPCs isolated from the bone marrow of 5-FU treated mice was compared in the same experiment. 5-FU depletes the bone marrow of cycling cells thus enriching it in non-cycling progenitors. Cells were transduced on two consecutive days as described in section 4.1 with the constitutive MLL-ENL expression construct pMSCV-M/E or the empty vector pMSCV-neo. In addition, co-transductions were performed with the conditional MLL-ENL construct pMSCV-TRE-M/E and the inducer pMSCV-tTA. Concentrated pMSCV-tTA virus was used for each co-transduction. After two rounds of transduction the cells were plated in methylcellulose (M3434 supplemented with GM-SCF) with G418 selection.

The constitutive MLL-ENL expression construct pMSCV-M/E was able to immortalise E12 Sca-1$^+$ c-Kit$^+$ HPCs (Figure 5.1A). Several of the primary colonies

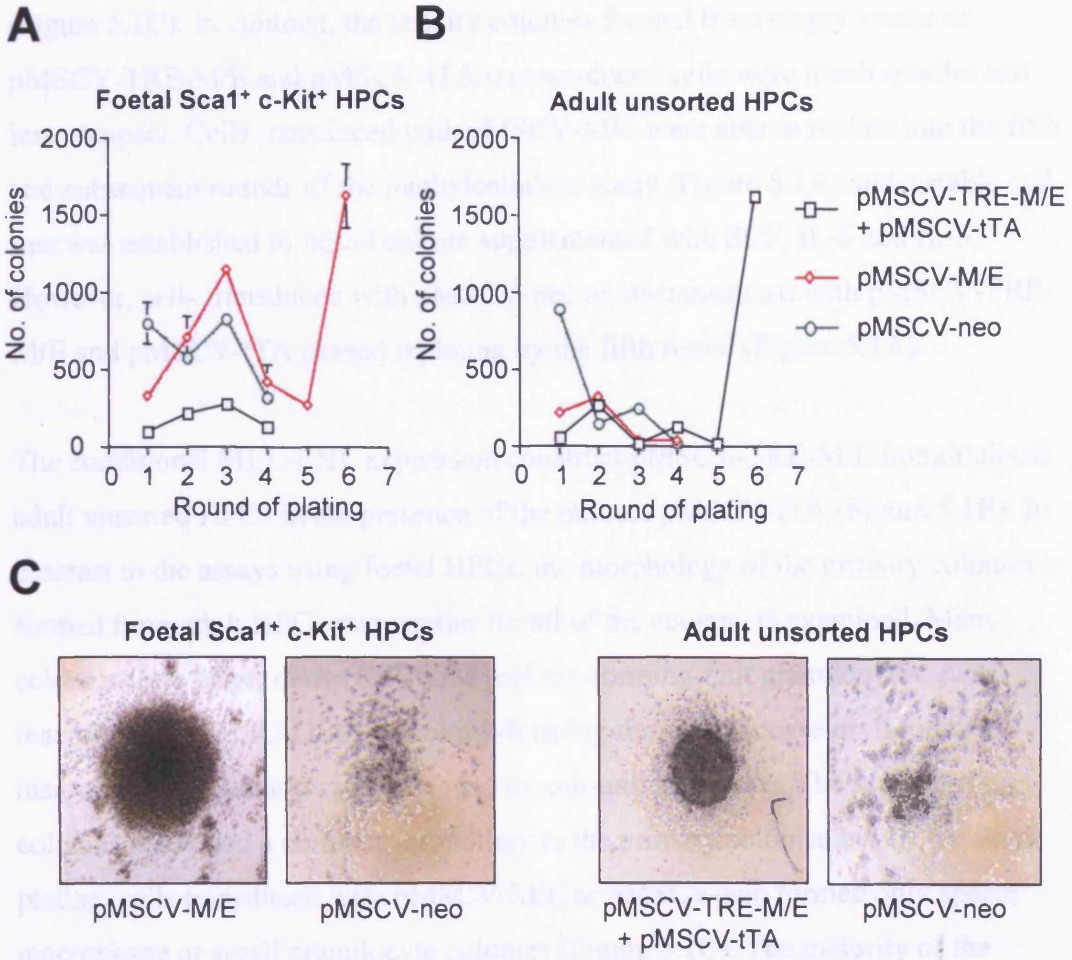

Figure 5.1 The conditional MLL-ENL expression construct can immortalise unsorted adult HPCs but not Sca-1$^+$ c-Kit$^+$ foetal HPCs. A) Sca-1$^+$ c-Kit$^+$ HPCs isolated from E12 foetal liver or **B)** unsorted HPCs isolated from the bone marrow of 5-FU treated mice were co-transduced with the conditional MLL-ENL expression construct pMSCV-TRE-M/E and pMSCV-tTA. Cells were also transduced with the constitutive MLL-ENL expression construct pMSCV-M/E or the empty vector pMSCV-neo. The transduced cells were cultured in methylcellulose under conditions that promoted myeloid development and serial replating assays were performed. The graphs show the number of colonies formed per 10^4 cells plated in each round of the assay following transduction with pMSCV-TRE-M/E and pMSCV-tTA (black squares), pMSCV-M/E (red diamonds) and pMSCV-neo (green circles). The mean and SD of duplicate cultures are shown in **A. C)** The morphology of colonies formed in the third round of the assays shown in **A** and **B** (original magnification x 40).

formed from pMSCV-M/E transduced cells were very large, round and compact, similar to the immortalised colonies observed in previous assays using c-Kit$^+$ Ter-119$^-$ HPCs. By the third round virtually all of the colonies were of this phenotype (Figure 5.1C). In contrast, the tertiary colonies formed from empty vector or pMSCV-TRE-M/E and pMSCV-tTA co-transduced cells were much smaller and less compact. Cells transduced with pMSCV-M/E were able to replate into the fifth and subsequent rounds of the methylcellulose assay (Figure 5.1A) and a stable cell line was established in liquid culture supplemented with SCF, IL-6 and IL-3. However, cells transduced with pMSCV-neo or co-transduced with pMSCV-TRE-M/E and pMSCV-tTA ceased replating by the fifth round (Figure 5.1A).

The conditional MLL-ENL expression construct pMSCV-TRE-M/E immortalised adult unsorted HPCs in the presence of the inducer pMSCV-tTA (Figure 5.1B). In contrast to the assays using foetal HPCs, the morphology of the primary colonies formed from adult HPCs was similar for all of the constructs examined. Many colonies were large, dense CFU-GM (colony-forming-unit granulocyte-macrophage) or CFU-GEMM (colony-forming-unit granulocyte-erythrocyte-macrophage-megakaryocyte) of round or non-uniform shape. The secondary colonies possessed a similar morphology to the primary colonies but by the third plating, cells transduced with pMSCV-M/E or pMSCV-neo formed only sparse macrophage or small granulocyte colonies (Figure 5.1C). The majority of the colonies formed from pMSCV-TRE-M/E and pMSCV-tTA co-transduced cells were of a similar morphology. However, one of the colonies formed from pMSCV-TRE-M/E and pMSCV-tTA co-transduced cells was large, round and dense (Figure 5.1C). This colony replated into the fourth and subsequent rounds of the methylcellulose assay and a stable cell line was established in liquid culture. Cells transduced with either the empty vector or the constitutive construct pMSCV-M/E exhausted their proliferative capacity by the fourth round of replating (Figure 5.1B).

5.2 The constitutive and conditional MLL-ENL expression constructs do not immortalise the same purified populations of adult HPCs.

In previous experiments, the constitutive MLL-ENL expression construct pMSCV-M/E immortalised c-Kit$^+$ Ter-119$^-$ foetal HPCs (2/6 experiments) and Sca-1$^+$ c-Kit$^+$ foetal HPCs (1/1 experiment) while the conditional MLL-ENL expression construct

pMSCV-TRE-M/E, either alone or in combination with pMSCV-tTA, did not (Figures 4.1 and 5.1A). However, the conditional MLL-ENL expression construct in combination with tTA, was able to immortalise unsorted adult HPCs (5/5 experiments) but the constitutive construct was not (Figure 5.1B). The ability of these constructs to immortalise purified populations of adult HPCs was examined by isolating lin^- and c-Kit^+ populations from the bone marrow of 5-FU treated mice by magnetic activated cell sorting (MACS). Transductions were performed as described in section 5.1.

The constitutive MLL-ENL expression construct pMSCV-M/E was able to immortalise c-Kit^+ adult HPCs (Figure 5.2A). Many of the tertiary colonies formed from pMSCV-M/E transduced cells were of the immortalised phenotype (Figure 5.2C). These colonies replated into subsequent rounds and a stable cell line was established in liquid culture. However, immortalisation was not observed using the conditional MLL-ENL expression construct either alone or in combination tTA (Figure 5.2A).

The conditional MLL-ENL expression construct pMSCV-TRE-M/E in combination with the inducer tTA was able to immortalise lin^- adult HPCs (Figure 5.2B). Many of the tertiary colonies formed from pMSCV-TRE-M/E and pMSCV-tTA co-transduced cells were of the immortalised phenotype (Figure 5.2C). These colonies replated into subsequent rounds and a stable cell line was established in liquid culture. However, immortalisation was not observed using the constitutive MLL-ENL expression construct (Figure 5.2B). The ability of the conditional construct to immortalise lin^- HPCs in the absence of tTA expression was not addressed in this experiment due to the low numbers of lin^- sorted cells obtained. In summary, the constitutive MLL-ENL expression construct was able to immortalise c-Kit^+ HPCs bit not lin^- HPCs and the conditional MLL-ENL expression construct in combination with tTA, was able to immortalise lin^- HPCs but not c-Kit^+ HPCs. The reason why the conditional and constitutive constructs failed to immortalise the same purified population of progenitors is not clear.

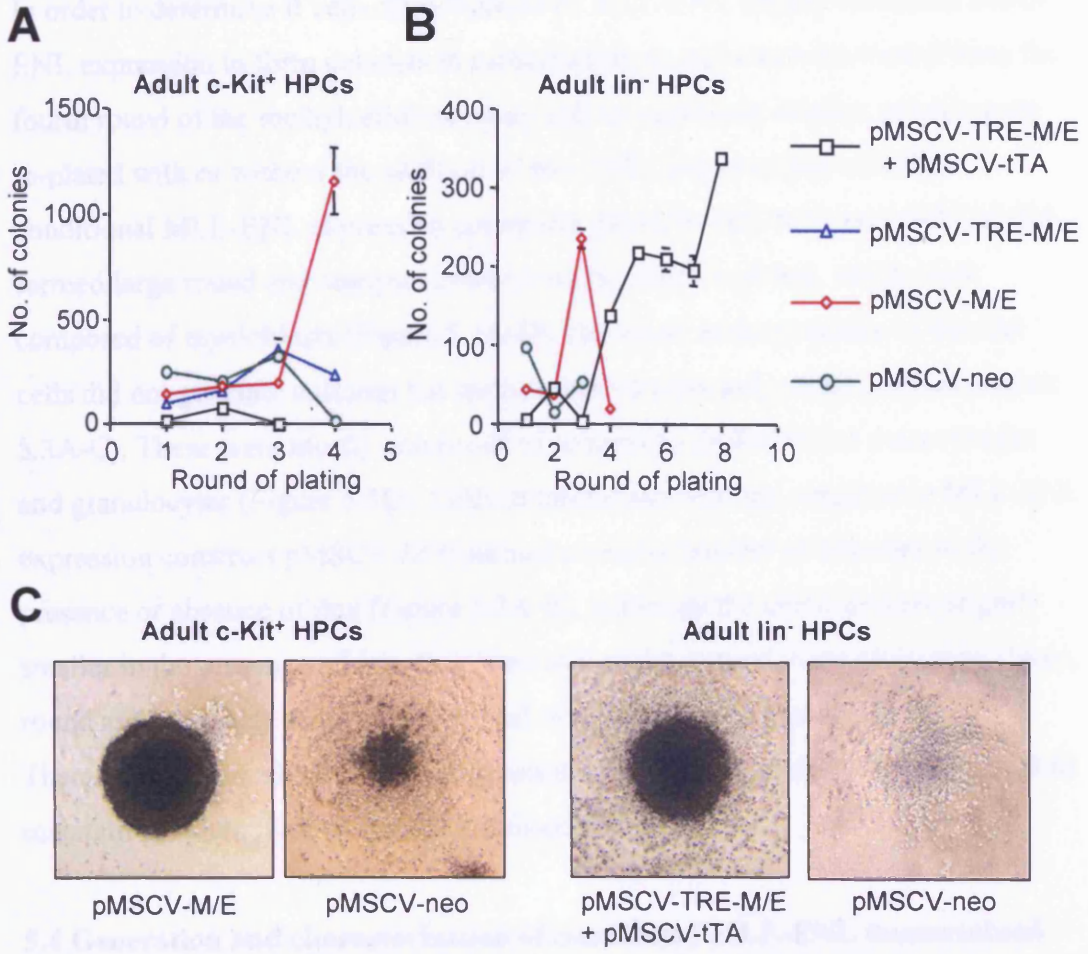

Figure 5.2 The conditional and constitutive MLL-ENL expression constructs do not immortalise the same population of purified adult HPCs. A) c-Kit$^+$ or **B)** lin$^-$ HPCs isolated from the bone marrow of 5-FU treated mice were co-transduced with the conditional MLL-ENL expression construct pMSCV-TRE-M/E and the inducer pMSCV-tTA. Cells were also transduced with the constitutive MLL-ENL expression construct pMSCV-M/E or the empty vector pMSCV-neo. c-Kit$^+$ HPCs were also transduced with the conditional construct in the absence of pMSCV-tTA. The transduced cells were cultured in methylcellulose under conditions that promoted myeloid development and serial replating assays were performed. The graphs show the number of colonies formed per 10^4 cells plated in each round of the assay following transduction with pMSCV-TRE-M/E and pMSCV-tTA (black squares), pMSCV-TRE-M/E alone (blue triangles), pMSCV-M/E (red diamonds) or pMSCV-neo (green circles). The mean and SD of duplicate cultures are shown in **A**. **C)** The morphology of colonies formed in the third round of the assays shown in **A** and **B** (original magnification x 40).

5.3 Continued MLL-ENL expression is required to maintain the immortalised phenotype *in vitro*.

In order to determine if cells immortalised by MLL-ENL require continued MLL-ENL expression to form colonies in methylcellulose, cells were harvested from the fourth round of the methylcellulose assay and an equivalent number of cells were re-plated with or without the addition of dox. Cells immortalised with the conditional MLL-ENL expression constructs pMSCV-TRE-M/E and pMSCV-tTA formed large round and compact colonies in the absence of dox, which were composed of myeloblasts (Figure 5.3A-D). However, in the presence of dox the cells did not generate colonies but instead formed extremely small clusters (Figure 5.3A-C). These were mostly composed of terminally differentiated macrophages and granulocytes (Figure 5.3D). Cells immortalised with the constitutive MLL-ENL expression construct pMSCV-M/E formed a similar number of colonies in the presence or absence of dox (Figure 5.3A-B). Although the colonies were slightly smaller in the presence of dox, they were still of the immortalised phenotype (large, round and compact) and were composed of myeloblasts (Figure 5.3C-D). Therefore, continued MLL-ENL expression is required for colony formation and to maintain the phenotype of the immortalised cells.

5.4 Generation and characterisation of conditional MLL-ENL immortalised cell lines.

Five conditional cell lines were generated from HPCs co-transduced with pMSCV-TRE-M/E and pMSCV-tTA in five independent experiments. However, only three (TRE-ME2, TRE-ME3 and TRE-ME6) were characterised in detail. The constitutive cell line (c-ME1) was generated from HPCs transduced with pMSCV-M/E. TRE-ME2 and TRE-ME6 were derived from unsorted bone marrow, TRE-ME3 was derived from lin^- bone marrow and c-ME1 was derived from c-Kit^+ bone marrow. All were generated using unconcentrated MLL-ENL virus apart from TRE-ME6 in which both the pMSCV-TRE-M/E and the pMSCV-tTA virus were concentrated.

All of the cell lines proliferated rapidly in liquid culture, with c-ME1 showing the highest rate of proliferation (Figure 5.4A). TRE-ME3 proliferated at a slower rate that the other cell lines at first (Figure 5.4A). However, after a few months in liquid

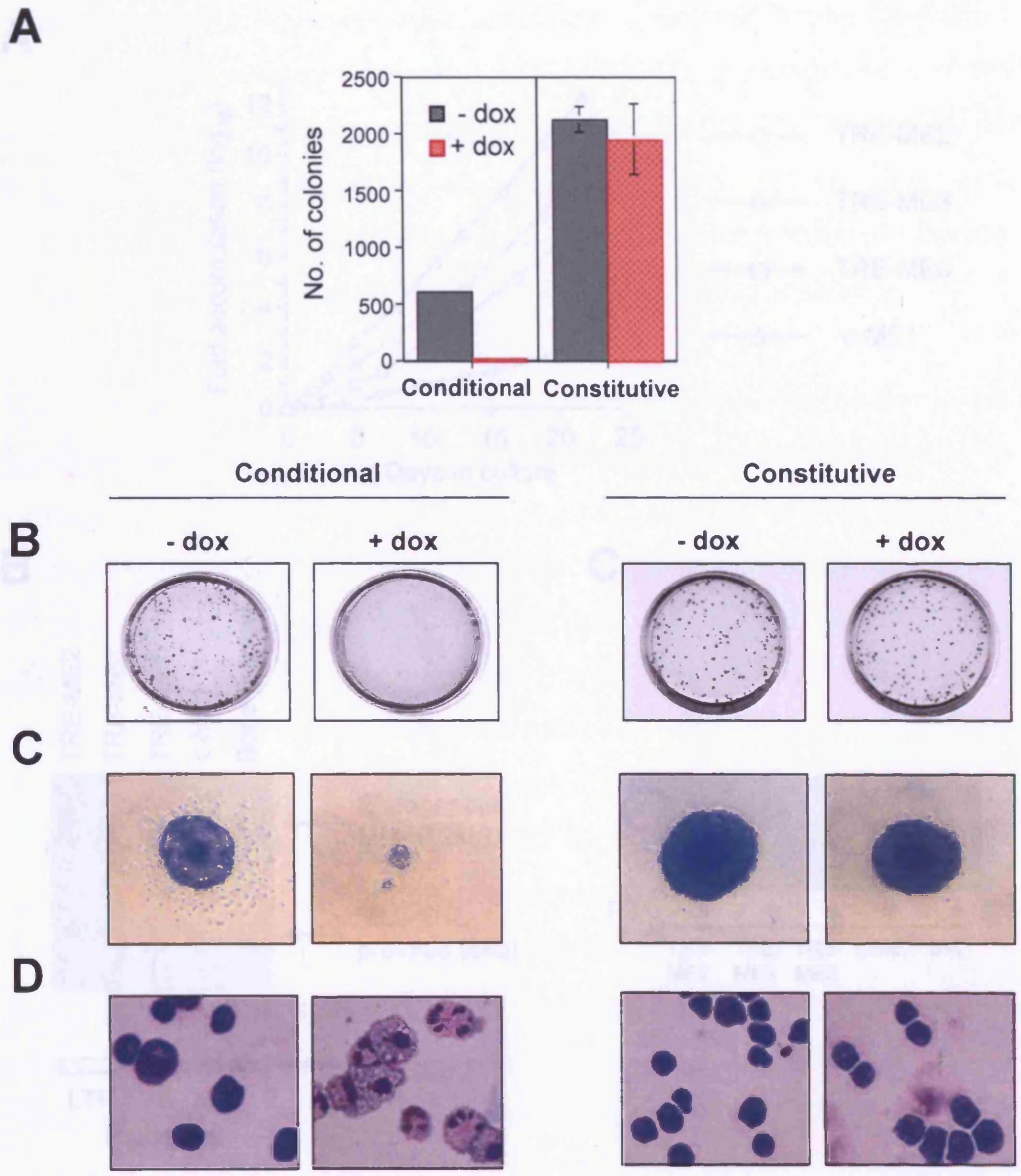

Figure 5.3 MLL-ENL is required to maintain as well as initiate immortalisation of HPCs. Cells transduced with either the conditional MLL-ENL expression construct (pMSCV-TRE-M/E) and the inducer pMSCV-tTA or the constitutive MLL-ENL expression construct (pMSCV-M/E) were harvested from the fourth round of the methylcellulose assay and replated into a subsequent round with or without the addition of dox. **A)** The number of colonies formed per 10^4 cells plated after 7 days is shown. The plot shows the mean and SD of duplicate cultures. **B)** INT stains of the fifth round methylcellulose cultures after culture in the presence or absence of dox for 7 days. **C)** Typical morphology of the colonies (original magnification, x 40) and **D)** typical morphology of the cells (original magnification, x 400) harvested from the fifth round of the assay after culture in the presence or absence of dox for 7 days. Cells were visualised by cytospin preparation followed by May-Grunwald-Giemsa staining.

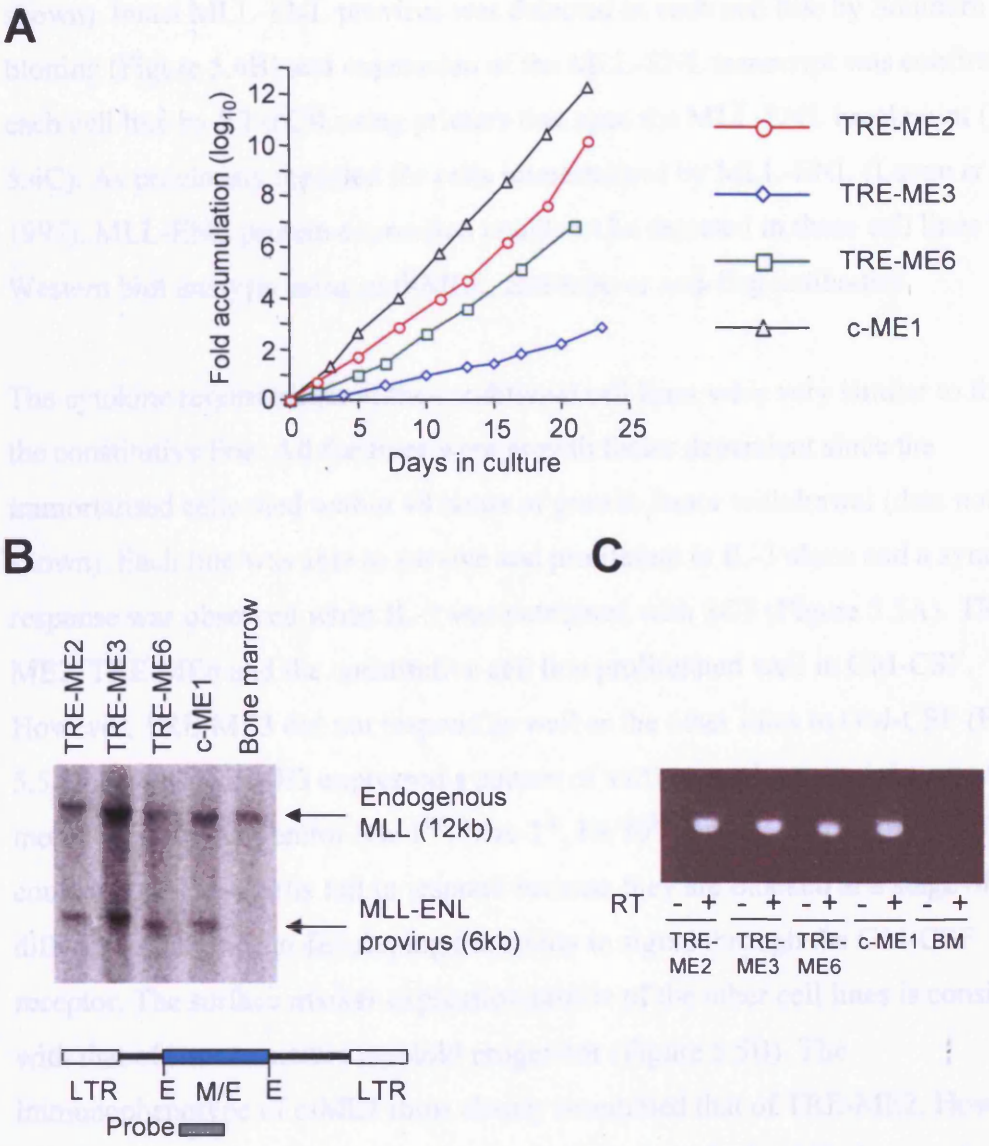

Figure 5.4 Generation of MLL-ENL immortalised cell lines. Immortalised cell lines were generated by placing cells harvested from the third round of the methylcellulose re-plating assay in liquid culture supplemented with SCF, IL-6 and IL-3. **A)** The graph shows the log of the fold accumulation in cell number of TRE-ME2 (red circles), TRE-ME3 (blue diamonds), TRE-ME6 (green squares) and c-ME1 (black triangles). **B)** Southern blot analysis of genomic DNA isolated from the cell lines showing integration of the MLL-ENL provirus. Untransduced bone marrow was used as a negative control. The indicated N-terminal MLL cDNA fragment was used as a probe to detect the 6-kilobase proviral band following *EcoRI* digestion of genomic DNA. The probe detects a fragment of the endogenous MLL gene (top arrow) and the MLL-ENL provirus (bottom arrow). E indicates *EcoRI*. **C)** Reverse-transcription (RT)-PCR analysis of total RNA isolated from the cell lines demonstrating expression of the MLL-ENL transcript. Untransduced bone marrow (BM) was used as a negative control. Negative control reactions were performed in the absence of reverse transcriptase (RT).

culture its rate of proliferation increased to match that of TRE-ME2 (data not shown). Intact MLL-ENL provirus was detected in each cell line by Southern blotting (Figure 5.4B) and expression of the MLL-ENL transcript was confirmed in each cell line by RT-PCR using primers that span the MLL-ENL breakpoint (Figure 5.4C). As previously reported for cells immortalised by MLL-ENL (Lavau *et al.*, 1997), MLL-ENL protein expression could not be detected in these cell lines by Western blot analysis using anti-MLL, anti-myc or anti-flag antibodies.

The cytokine requirements of the conditional cell lines were very similar to that of the constitutive line. All the lines were growth factor dependent since the immortalised cells died within 48 hours of growth factor withdrawal (data not shown). Each line was able to survive and proliferate in IL-3 alone and a synergistic response was observed when IL-3 was combined with SCF (Figure 5.5A). TRE-ME2, TRE-ME6 and the constitutive cell line proliferated well in GM-CSF. However, TRE-ME3 did not respond as well as the other lines to GM-CSF (Figure 5.5A). Since TRE-ME3 expressed a pattern of surface markers reminiscent of a more immature progenitor (Gr-1^{int}, Mac-1^{lo}, F4/80^{lo} and c-Kit^{+}) (Figure 5.5B), it could be that these cells fail to respond because they are blocked at a stage of differentiation prior to developing the ability to signal through the GM-CSF receptor. The surface marker expression profile of the other cell lines is consistent with that of a more mature myeloid progenitor (Figure 5.5B). The immunophenotype of c-ME1 most closely resembled that of TRE-ME2. However, c-ME1 expressed lower levels of Gr-1 and F4/80 than TRE-ME2. Hence, the cell surface marker expression profile of c-ME1 is consistent with that of a slightly more immature myeloid progenitor than TRE-ME2. TRE-ME6 expressed lower levels of Gr-1 and c-Kit and higher levels of Mac-1 and F4/80 than any of the other cell lines (Figure 5.5B). This data suggests that TRE-ME6 is further engaged along the myeloid differentiation pathway than the other cell lines.

5.5 MLL-ENL immortalised cell lines cease proliferating upon loss of MLL-ENL expression.

In order to establish if MLL-ENL expression could be regulated by dox in the conditional cell lines a Q-PCR strategy was developed to measure the levels of MLL-ENL transcript expression in these cell lines (section 2.24). Culture of the

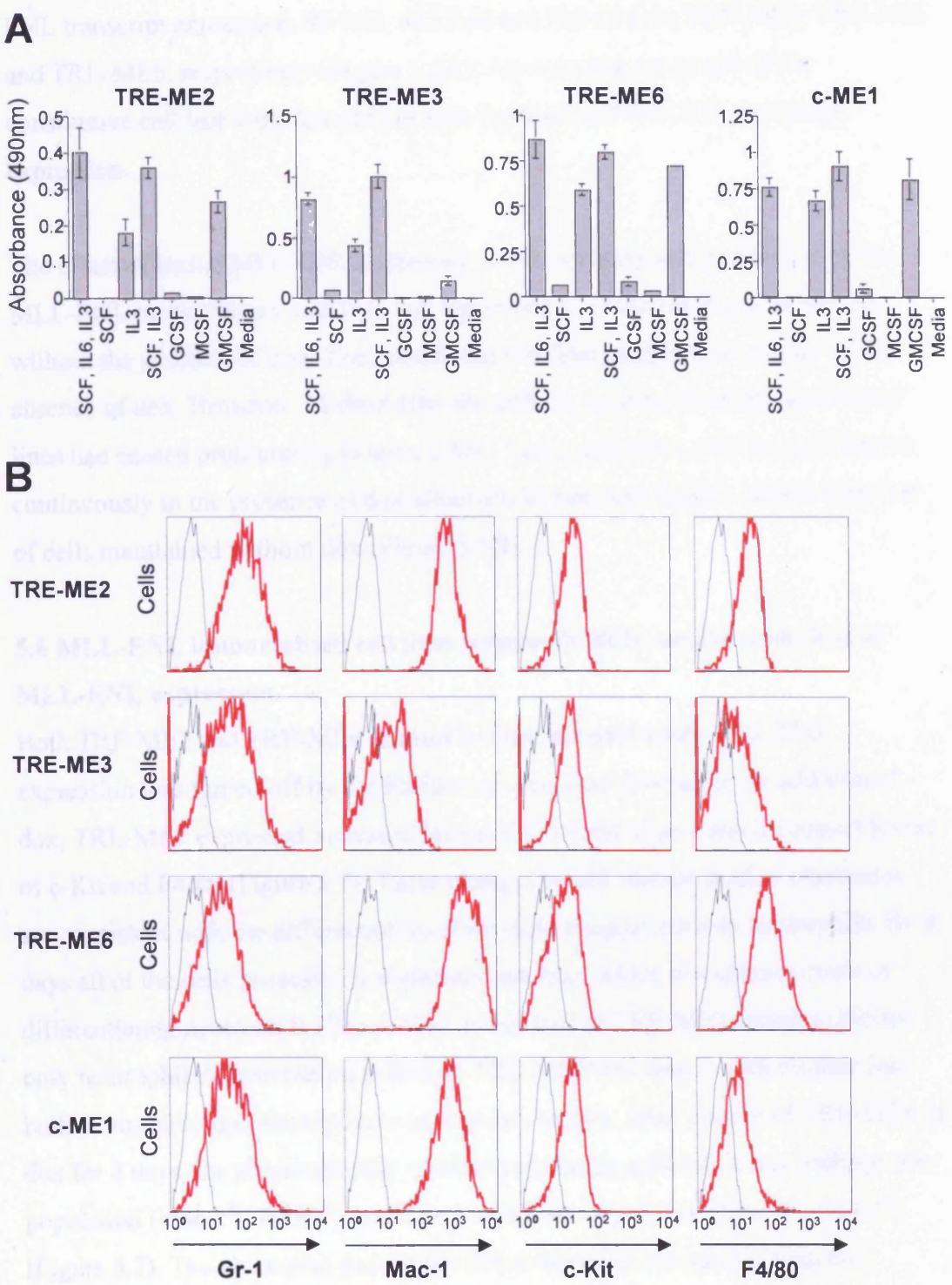

Figure 5.5 Characterisation of MLL-ENL immortalised cell lines. A) The graphs show the absorbance from MTS assays performed on the cell lines following culture with the indicated cytokines for 48 hours. Plots show the mean and SD of triplicate absorbance readings. **B)** Flow cytometric analysis of cell surface antigen expression of the cell lines. Thick red lines represent the expression profile of the indicated antigen and thin black lines represent the appropriate isotype control.

conditional cell lines with dox for 24 hours resulted in a large decrease in MLL-ENL transcript expression, 93 fold, 482 fold and 705 fold for TRE-ME2, TRE-ME3 and TRE-ME6, respectively (Figure 5.6A). As expected, treatment of the constitutive cell line with dox did not alter the level of MLL-ENL transcript expression.

The effect of losing MLL-ENL expression on the viability and phenotype of the MLL-ENL immortalised cell lines was examined by culturing the cells with or without the addition of dox. The conditional cell lines proliferated rapidly in the absence of dox. However, 10 days after the addition of dox, all of the conditional lines had ceased proliferating (Figure 5.6B). The constitutive cell line proliferated continuously in the presence of dox although the rate was slightly slower than that of cells maintained without dox (Figure 5.6B).

5.6 MLL-ENL immortalised cell lines terminally differentiate upon loss of MLL-ENL expression.

Both TRE-ME2 and TRE-ME6 terminally differentiated when MLL-ENL expression was turned off by the addition of dox. Four days after the addition of dox, TRE-ME2 expressed increased levels of Gr-1 and Mac-1 and decreased levels of c-Kit and F4/80 (Figure 5.7). These changes in cell surface marker expression are consistent with the differentiation of myeloid progenitors into neutrophils. By 8 days all of the cells possessed a segmented nucleus, which is a characteristic of differentiating neutrophils (Figure 5.8). In contrast to TRE-ME2 which exhibited only neutrophil differentiation potential, TRE-ME6 was able to differentiate into both neutrophils and macrophages in response to dox. After culture of TRE-ME6 in dox for 4 days, the phenotypically uniform population split into a macrophage sub-population (Mac-1^{hi}, F4/80^{hi}) and a neutrophil sub-population (Mac-1^{+}, F4/80^{lo}) (Figure 5.7). The neutrophil population differentiated more quickly than the macrophage population and after culture in dox for 8 days the neutrophils had terminally differentiated and died, since only mature macrophages were observed (Figure 5.8).

In contrast to TRE-ME2 and TRE-ME6, TRE-ME3 did not terminally differentiate in response to dox. An increase in Gr-1 expression and a decrease in c-Kit

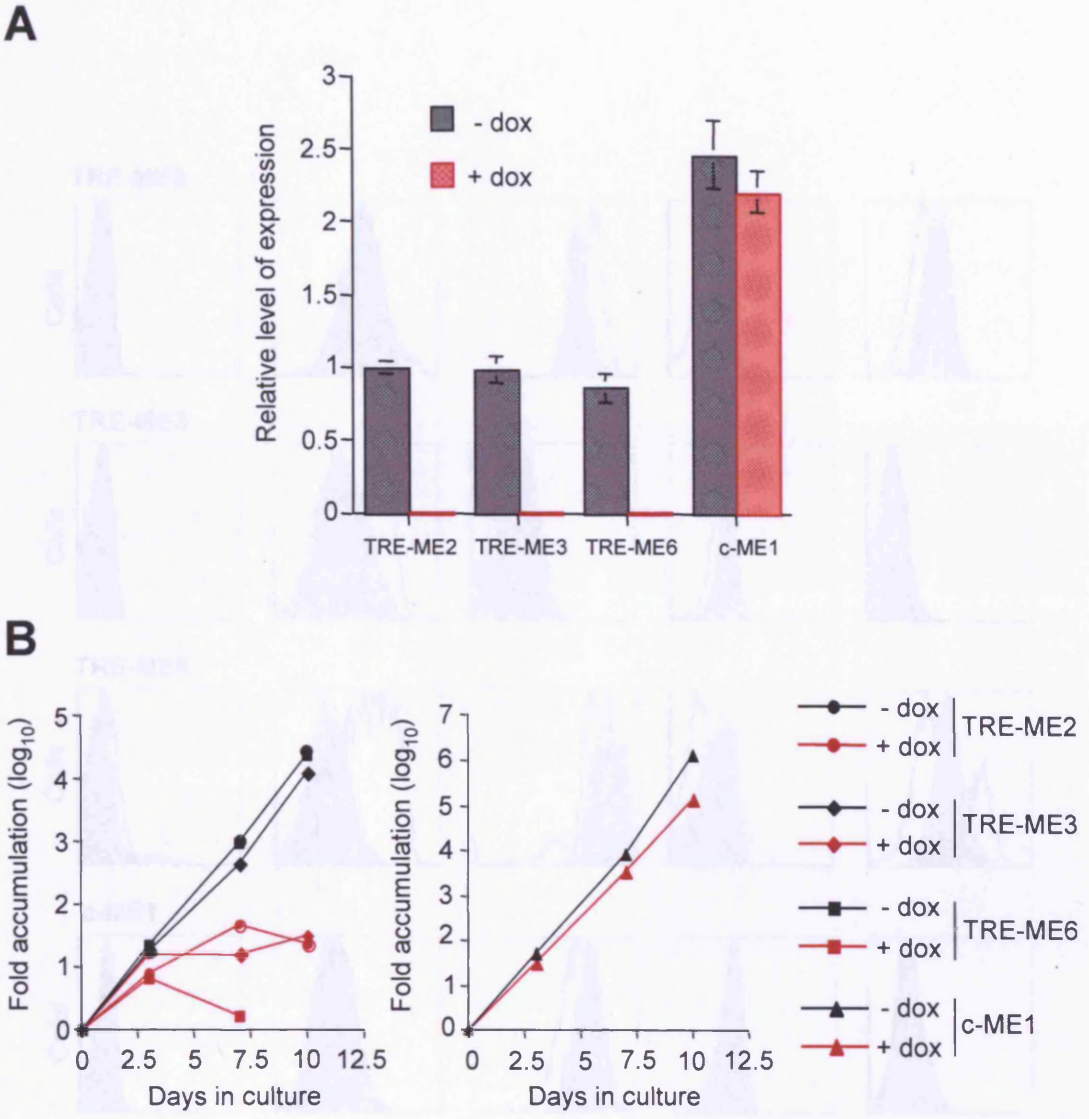

Figure 5.6 MLL-ENL cell lines cease proliferating upon loss of MLL-ENL expression. A) The graph shows the relative level of MLL-ENL mRNA expression in TRE-ME2, TRE-ME3, TRE-ME6 and c-ME1 following treatment with (red columns) or without dox (black columns) for 24 hours. Total RNA was used to make cDNA for Q-PCR analysis and data was normalised to GAPDH. The mean and SD of triplicate measurements are shown. **B)** The graphs show the log of the fold accumulation in cell number following maintenance of TRE-ME2 (circles), TRE-ME3 (diamonds), TRE-ME6 (squares) and c-ME1 (triangles) with (red lines) or without dox (black lines).

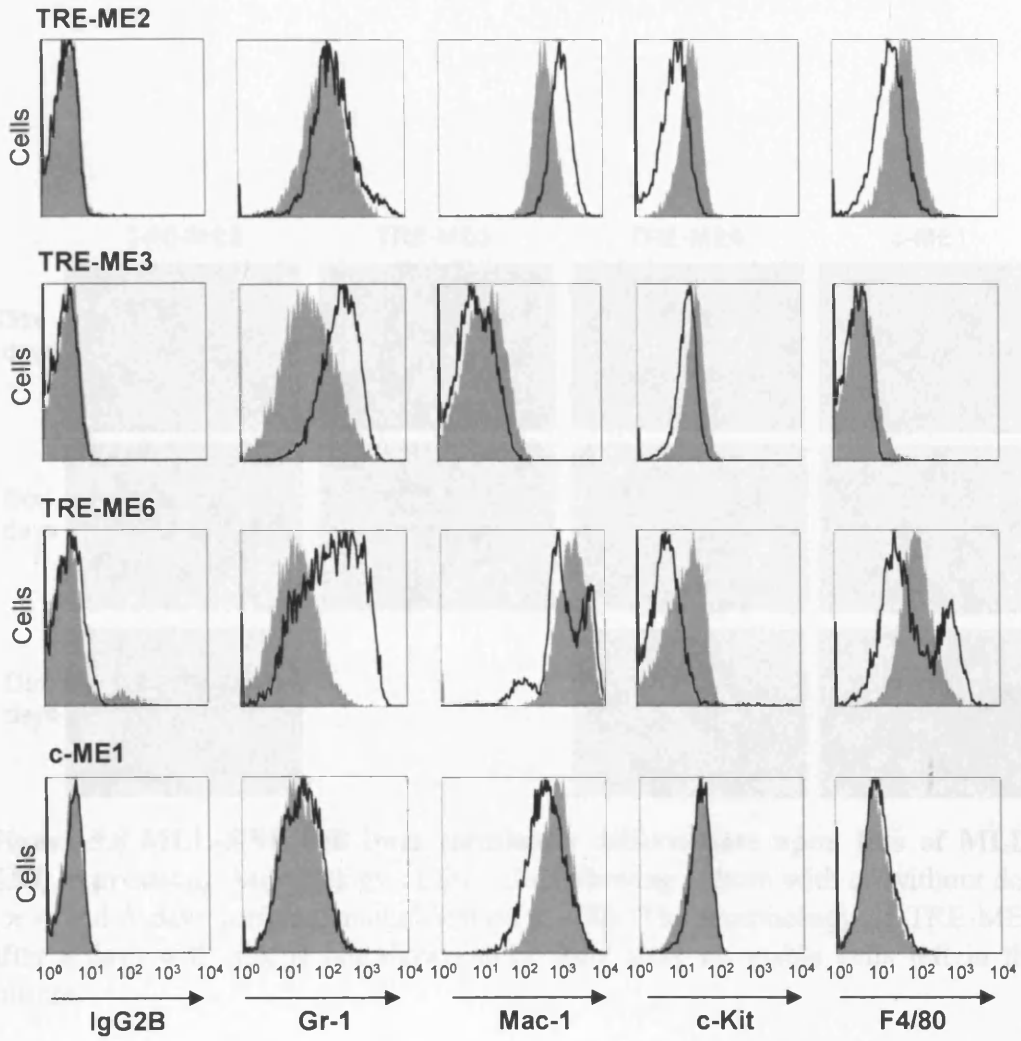

Figure 5.7 MLL-ENL cell lines terminally differentiate upon loss of MLL-ENL expression. Flow cytometric analysis of cell surface antigen expression following culture of the cell lines with (thick black lines) or without dox (shaded plots) for 4 days.

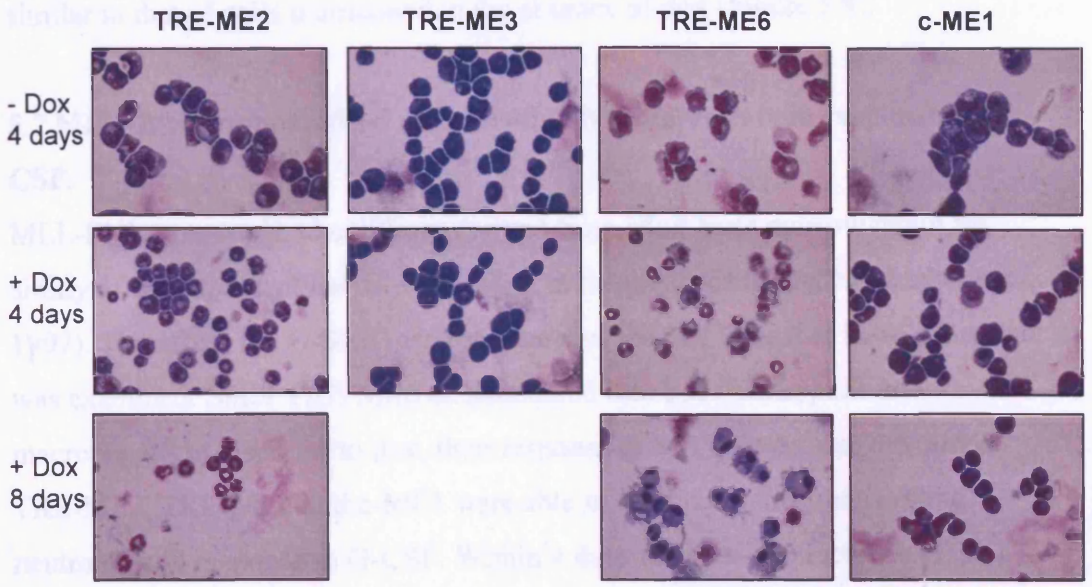

Figure 5.8 MLL-ENL cell lines terminally differentiate upon loss of MLL-ENL expression. Morphology of the cells following culture with or without dox for 4 and 8 days (original magnification x 400). The morphology of TRE-ME3 after 8 days with dox is not shown since there were no viable cells left in the culture.

expression were observed after culture in dox for 4 days which is consistent with neutrophil differentiation (Figure 5.7). However, at this time-point the majority of the cells were still myeloblasts, very few cells possessed a segmented nucleus (Figure 5.8). This is probably because TRE-ME3 is more immature than the other conditional cell lines and would therefore take longer to differentiate. However, instead of terminally differentiating into neutrophils, the cells died. After 8 days in the presence of dox, no viable cells were left in the culture (data not shown). Therefore, it seems that TRE-ME3 is able to initiate but not complete the process of differentiation. As expected, the constitutive cell line did not differentiate in response to dox. Eight days after the addition of dox the cellular morphology was similar to that of cells maintained in the absence of dox (Figure 5.8).

5.7 MLL-ENL immortalised cells terminally differentiate in response to G-CSF.

MLL-ENL immortalised cell lines derived from adult bone marrow retain the ability to undergo terminal differentiation in the presence of G-CSF (Lavau *et al.*, 1997). Therefore, the G-SCF responsiveness of the cell lines that I had generated was examined. Since TRE-ME6 differentiated into both neutrophils and macrophages in response to dox, their response to M-CSF was also examined. TRE-ME2, TRE-ME6 and c-ME1 were able to terminally differentiate into neutrophils in response to G-CSF. Within 4 days there was an increase in Gr-1 expression and a decrease in c-Kit expression (Figure 5.9). TRE-ME2 and c-ME1 expressed increased levels of Mac-1 after 4 days of G-CSF treatment. This is consistent with the differentiation of myeloid blasts into myelomonocytic cells (Lagasse and Weissman, 1996). However, the level of Mac-1 expressed by TRE-ME6 decreased after 4 days of G-CSF treatment (Figure 5.9). This is consistent with the fact that TRE-ME6 is further engaged along the myeloid differentiation pathway that the other cell lines (Figure 5.5) and that Mac-1 expression normally decreases at late stages of neutrophil differentiation (Lagasse and Weissman, 1996). By 4 days TRE-ME6 had completely differentiated into neutrophils whereas c-ME1 and TRE-ME2 took another 4 days to reach a similar stage of differentiation (Figure 5.10). In contrast to the other cell lines, TRE-ME3 died within 48 hours when cultured in G-CSF (data not shown), which is consistent with its inability to terminally differentiate into neutrophils in response to dox.

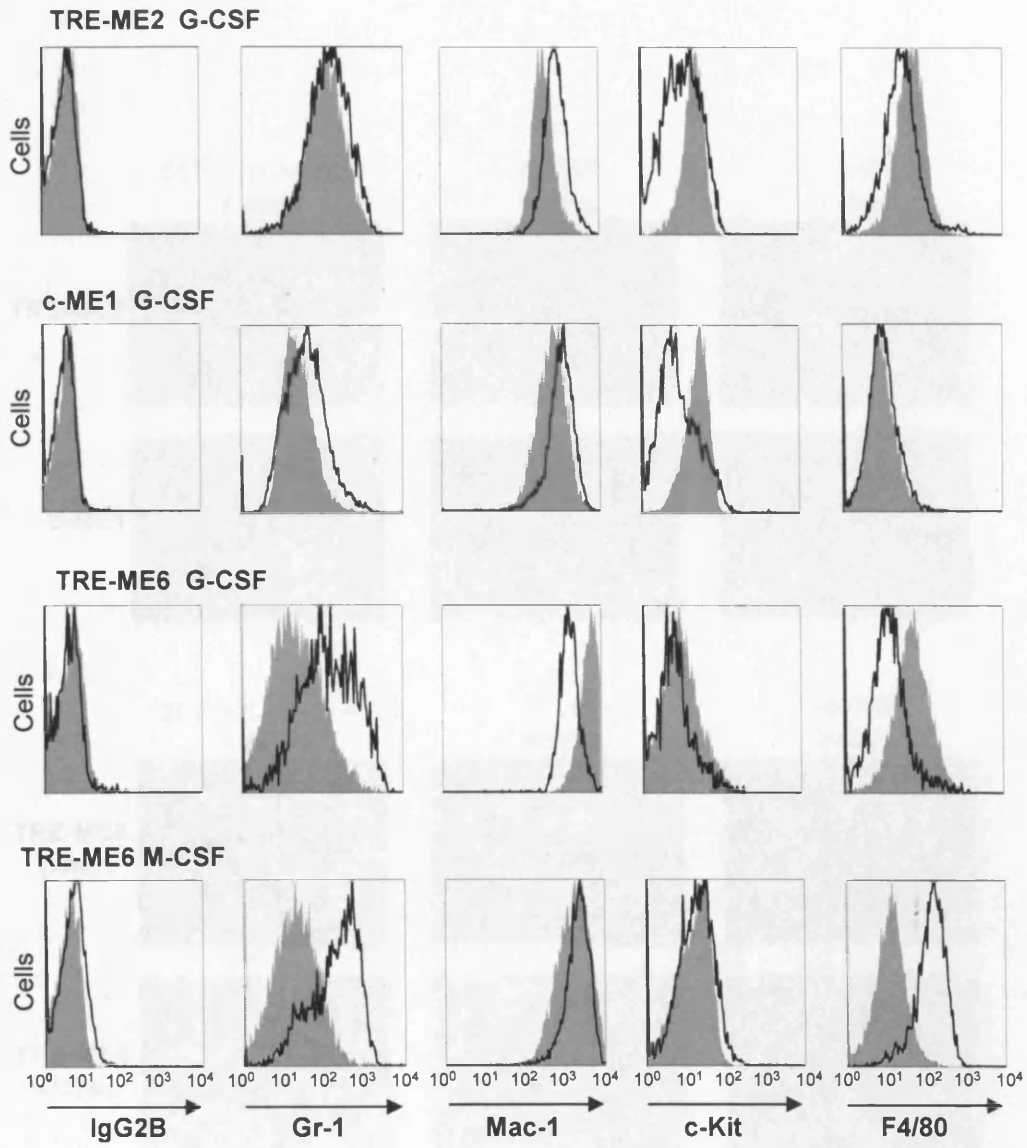

Figure 5.9 MLL-ENL cell lines differentiate in response to G-CSF. Flow cytometric analysis of cell surface antigen expression following culture of the cell lines in either G-CSF or M-CSF. The cells treated with G-CSF were analysed after 4 days and the cells treated with M-CSF were analysed after 3 days. Shaded plots represent expression of the indicated antigen following culture with SCF, IL6 and IL-3. Thick black lines represent expression of the antigen following culture in either G-CSF or M-CSF.

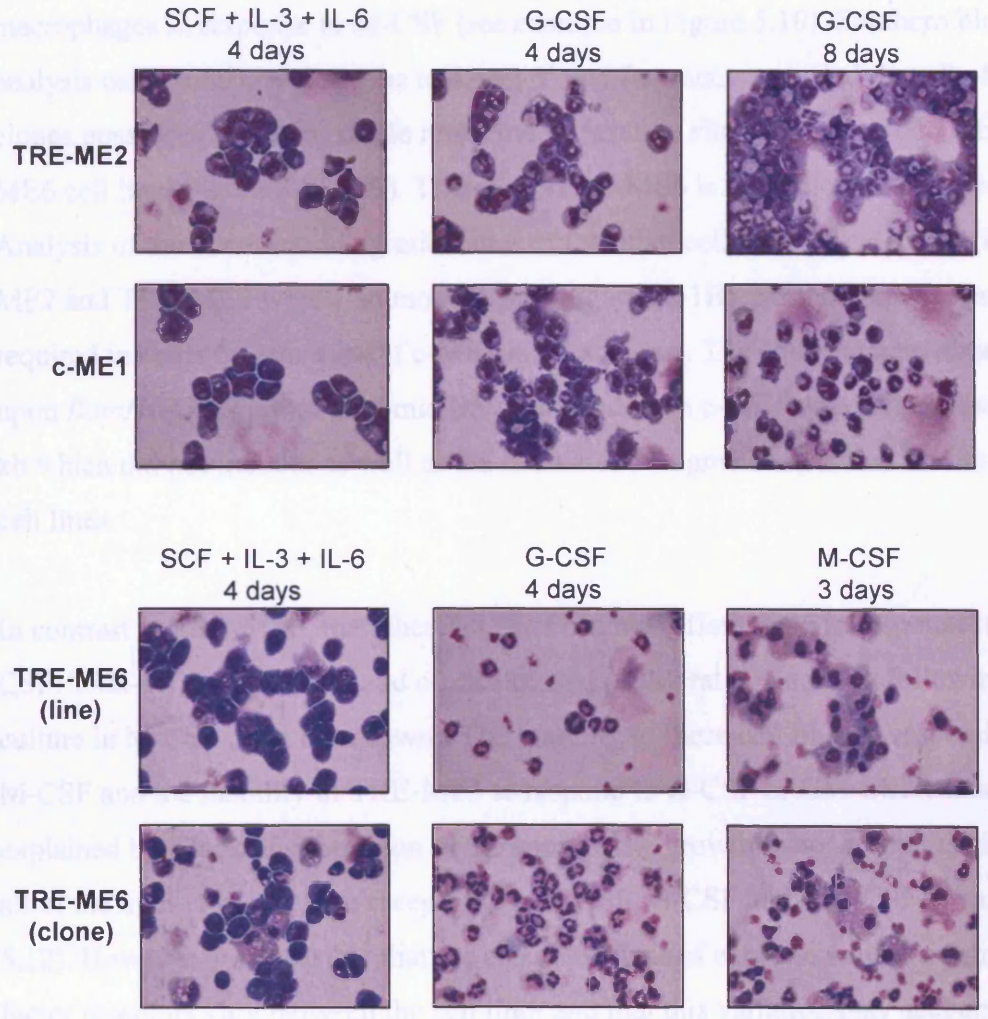

Figure 5.10 MLL-ENL cell lines differentiate in response to G-CSF. Morphology of the cell lines and a representative clone of TRE-ME6 following culture with the indicated cytokines (original magnification x 400).

TRE-ME6 was the only cell line able to differentiate into macrophages in response to M-CSF. After 4 days the cells expressed increased levels of Mac-1, Gr-1 and F4/80 (Figure 5.9) and possessed a large and vacuolar cytoplasm (Figure 5.10). The TRE-ME6 cell line was cloned in order to determine whether it was comprised of different unipotent clones capable of differentiation into either neutrophils or macrophages, or whether it was truly bipotent. All five of the clones derived from this cell line were able to differentiate into neutrophils in response to G-CSF and macrophages in response to M-CSF (see example in Figure 5.10). Southern blotting analysis using a neomycin probe to detect 5' end fragments revealed that all of the clones possessed the same single retroviral integration site as the parental TRE-ME6 cell line (Figure 5.11A-B). Therefore, TRE-ME6 is monoclonal and bi-potent. Analysis of the retroviral integration sites of the other cell lines revealed that TRE-ME2 and TRE-ME3 were also monoclonal (Figure 5.11B). Further experiments are required in order to determine if c-ME1 is monoclonal. The end fragment obtained upon *BamH1* digestion of genomic DNA isolated from c-ME1 was greater than 11 kb which did not transfer as well as the shorter end fragments obtained for the other cell lines.

In contrast to TRE-ME6, the other cell lines did not differentiate in response to M-CSF. TRE-ME2, TRE-ME3 and c-ME1 ceased proliferating and died following culture in M-CSF (data not shown). The inability of these cell lines to respond to M-CSF and the inability of TRE-ME3 to respond to G-CSF or GM-CSF cannot be explained by a lack of expression of the appropriate growth factor receptors since all of the lines expressed the receptors for M-CSF, G-CSF and GM-CSF (Figure 5.12). However, it is possible that the absolute levels of expression of these growth factor receptors vary between the cell lines and that this variation may account for the different cytokine responses observed. A more quantitative analysis such as Q-PCR should reveal if this is the case.

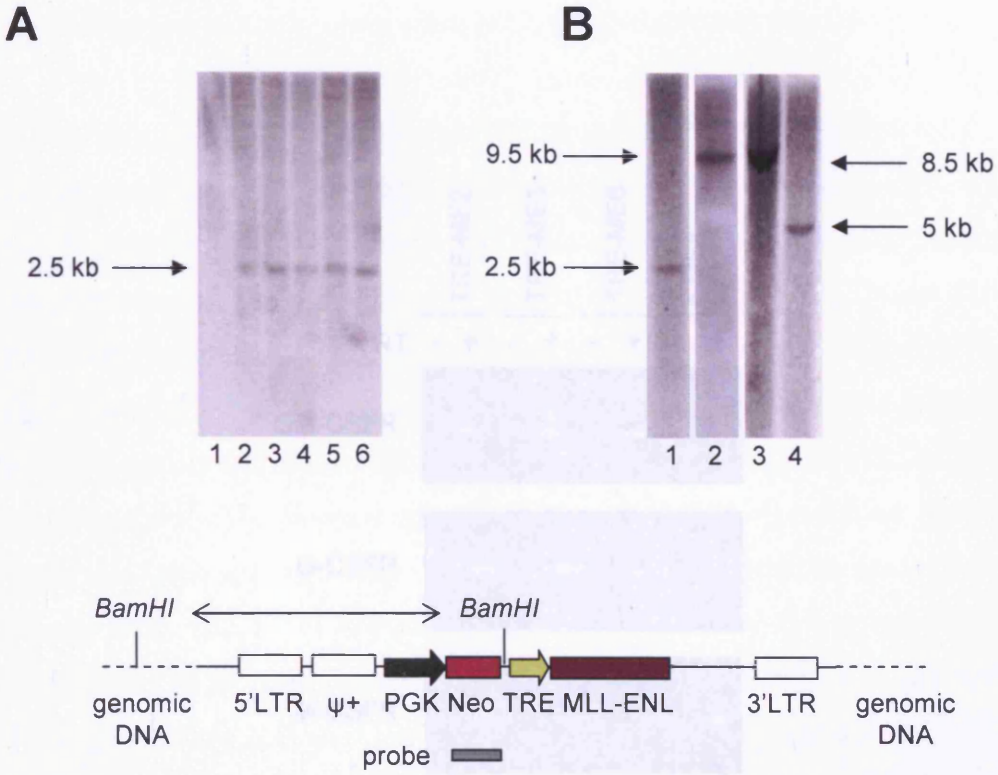

Figure 5.11 The conditional MLL-ENL cell lines are monoclonal. A) Southern blot analysis of genomic DNA isolated from 5 TRE-ME6 clones in lanes 2-6 respectively. Untransduced bone marrow was used as a negative control (lane 1). **B)** Southern blot analysis of genomic DNA isolated from the parental TRE-ME6 cell line (lane 1), TRE-ME2 (lane 2), TRE-ME3(A) (lane 3) and TRE-ME3(B) (lane 4). TRE-ME3(A) is the TRE-ME3 cell line analysed in this chapter. TRE-ME3(B) will be described in chapter 6. Genomic DNA was digested with *BamHI* which cuts just after the neomycin gene within the provirus. By probing the blot with a neomycin cDNA fragment, retroviral integration sites can be identified based on the proximity of the 5'LTR to the nearest endogenous *BamHI* site. Different integration sites would give rise to bands of different sizes. A single band suggests that the cell line has one integration and is therefore monoclonal. The arrows indicate the proviral integration sites.

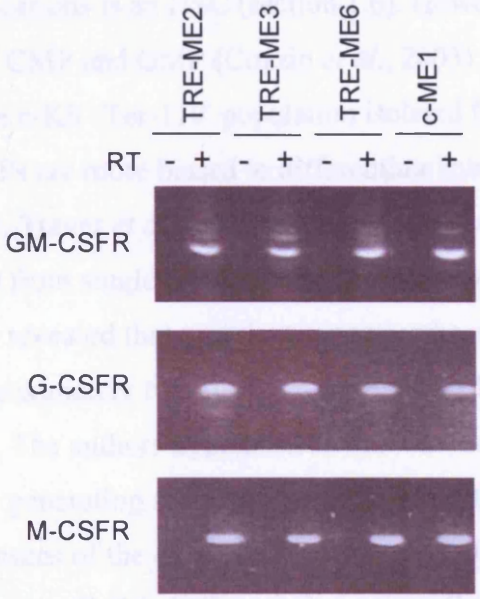

Figure 5.12 Expression of growth factor receptors by the MLL-ENL cell lines. The figure shows the expression of the granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) (upper panel), granulocyte-colony-stimulating factor receptor (G-CSFR) (middle panel) and macrophage-colony-stimulating factor receptor (M-CSFR) (lower panel) by each cell line. Transcript expression was analysed by RT-PCR using primers specific for each receptor. Negative control reactions were performed in the absence of reverse transcriptase (RT).

Discussion

We hypothesised that the c-Kit$^+$ Ter-119$^-$ progenitor population from E12 foetal liver may not be the best source of HPCs to generate MLL-ENL immortalised cell lines since the immortalisation observed using the constitutive MLL-ENL expression construct was not reproducible. Immortalisation was only observed in 2 out of 6 experiments using the constitutive MLL-ENL expression construct and was never observed using the conditional MLL-ENL expression construct.

A current hypothesis is that the target cell susceptible to transformation by chromosomal translocations is an HSC (section 1.6). However, MLL-ENL is also able to transform the CMP and GMP (Cozzio *et al.*, 2003). HSCs, CMPs and GMPs are present within the c-Kit$^+$ Ter-119$^-$ population isolated from E12 foetal liver. However, foetal CMPs are more biased to differentiate into MEPs than GMPs (Nicolini *et al.*, 1999; Traver *et al.*, 2001). Comparison of differential counts of colony types derived from single foetal CMPs (Traver *et al.*, 2001) and adult CMPs (Akashi *et al.*, 2000) revealed that megakaryocyte-erythroid production from the foetal CMP was approximately two-fold greater than that from the adult CMP (Traver *et al.*, 2001). The authors hypothesised that this was due to the critical role of the foetal CMP in generating sufficient numbers of erythrocytes to oxygenate the rapidly expanding tissues of the embryo (Traver *et al.*, 2001). Since the GMP rather than the MEP is a target of MLL-ENL transformation (Cozzio *et al.*, 2003), a purer population of foetal HSCs was isolated for retroviral transduction. The ability of MLL-ENL to immortalise foetal HSCs and unsorted adult HPCs was directly compared. The constitutive MLL-ENL expression construct successfully immortalised the HSC enriched population of E12 Sca-1$^+$ c-Kit$^+$ progenitors but the conditional MLL-ENL expression did not. In contrast, the conditional MLL-ENL expression construct in combination with the inducer tTA, successfully immortalised unsorted adult HPCs from 5-FU treated mice but the constitutive MLL-ENL expression construct did not.

Further experiments revealed that the constitutive and conditional constructs consistently failed to immortalise the same purified population of progenitors. The constitutive construct immortalised foetal and adult c-Kit$^+$ HPCs whereas the conditional construct immortalised unsorted and lin$^-$ adult HPCs from 5-FU treated

mice. A potential explanation for this observation is that the level of MLL-ENL expression required to initiate immortalisation may differ between populations of progenitors. It is possible that high levels of MLL-ENL expression are required to immortalise progenitor populations that are enriched in HSCs such as c-Kit$^+$ Ter-119$^-$ or Sca-1$^+$ c-Kit$^+$ foetal HPCs and adult c-Kit$^+$ HPCs. Lower levels of MLL-ENL expression may not facilitate immortalisation of these populations. If the LTR of pMSCV is capable of driving higher levels of MLL-ENL expression than the TRE, this hypothesis might explain the results. Indeed, quantitative analysis revealed that the constitutive cell line (c-ME1) which was derived from adult c-Kit$^+$ HPCs, expressed higher levels of the MLL-ENL transcript than all three conditional cell lines which were derived from adult lin$^-$ and unsorted HPCs. Therefore, it is possible that low levels of MLL-ENL can immortalise more mature populations of HPCs such as those found in the lin$^-$ and unsorted populations from adult bone marrow. Higher levels of MLL-ENL expression may be deleterious to these more mature populations. In accordance with this, it has been reported that high levels of MLL-fusion protein expression are incompatible with the survival of some cell types (Caslini *et al.*, 2000; Ayton and Cleary, 2001) (section 1.13).

Cells immortalised by MLL-ENL failed to self-renew in methylcellulose upon loss of MLL-ENL expression suggesting that continued MLL-ENL expression is required to maintain the immortalised phenotype *in vitro*. Furthermore, conditional myeloid cell lines were generated which either terminally differentiated or died upon switching off MLL-ENL expression by addition of dox. This result is consistent with previous studies in which haematopoietic progenitor cells were immortalised with an oestrogen-regulated conditional MLL-ENL fusion protein (Ayton and Cleary, 2003; Zeisig *et al.*, 2004). In the latter study immortalised myeloid cell lines were generated which were dependent on tamoxifen for their continued proliferation and survival and underwent terminal neutrophil differentiation following the withdrawal of tamoxifen. Two out of the three conditional cell lines I generated (TRE-ME2 and TRE-ME6), terminally differentiated into neutrophils upon loss of MLL-ENL expression. TRE-ME6 was bipotent, since it also exhibited macrophage differentiation potential in response to dox and clones were capable of neutrophil or macrophage differentiation in response to G-CSF or M-CSF, respectively. The bipotent nature of this cell line

suggests that the differentiation block imposed by MLL-ENL occurred prior to commitment to either the granulocyte or monocyte lineage, at the level of, or upstream of, the GMP. In contrast, TRE-ME2 and the constitutive cell line (c-ME1) were capable of neutrophil differentiation in response to G-CSF but lacked the ability to respond to M-CSF even though they expressed the M-CSF receptor. This might suggest that the differentiation block imposed by MLL-ENL in these cell lines is downstream of the GMP, after commitment to the granulocyte lineage. However, TRE-ME2 and c-ME1 possessed a more immature immunophenotype than TRE-ME6. It is possible that the differentiation block occurred prior to neutrophil commitment in TRE-ME2 and c-ME1 and that MLL-ENL may suppress macrophage differentiation to a greater extent in these cell lines. Interestingly, the third conditional cell line (TRE-ME3) lacked the ability to respond to both G-CSF and M-CSF and failed to complete differentiation upon loss of MLL-ENL expression. The phenotype of this cell line is consistent with that of a more immature myeloid progenitor than the other cell lines. It is possible that this cell line fails to undergo cytokine induced differentiation because MLL-ENL suppresses factors required for both neutrophil and macrophage differentiation. Hence, the more immature the progenitor immortalised by MLL-ENL, the stronger the differentiation block imposed. However, TRE-ME3 also failed to differentiate upon loss of MLL-ENL expression. Therefore, it is possible that this cell line has acquired other genetic changes such that it cannot differentiate and instead undergoes cell death upon loss of MLL-ENL expression. The fact that TRE-ME3 proliferated more slowly than the other cell lines at first but matched the rate of proliferation of the other cell lines after a few months in culture suggests that it may have acquired secondary mutations.

Immortalised MLL-ENL cell lines generated by retroviral transduction of purified populations of HSCs, CMPs and GMPs were found to be blocked at an identical stage of myelomonocytic development and induced AML in mice with identical latencies and phenotype (Cozzio *et al.*, 2003). The authors of this study state that the differentiation block imposed by MLL-ENL in these cells occurs at the unipotent monocytic progenitor stage. However, the present experiments and previous studies (Lavau *et al.*, 1997) show that MLL-ENL immortalised cells retain neutrophil differentiation capacity which would be incompatible with a block at the

level of the unipotent monocytic progenitor stage. Interestingly, the cell lines that I have generated do not possess identical immunophenotypes which suggests that MLL-ENL can arrest the differentiation of cells at different stages of myeloid development. I have also demonstrated that MLL-ENL can immortalise progenitors which retain both neutrophil and macrophage differentiation potential. Therefore, the differentiation arrest imposed by MLL-ENL can occur prior to lineage commitment.

Chapter 6 Identification of targets of MLL-ENL.

It has been previously suggested that *Hoxa7*, *Hoxa9* and *Meis-1* are targets of MLL-ENL (Zeisig *et al.*, 2004). However, it is not known if other members of the *Hoxa* cluster such as *Hoxa4*, *Hoxa5* and *Hoxa10*, which are frequently over-expressed in patients with 11q23 translocations (Armstrong *et al.*, 2002; Debernardi *et al.*, 2003; Rozovskaia *et al.*, 2003), are also targets of MLL-ENL. A current hypothesis is that MLL-fusion proteins immortalise HPCs by aberrantly maintaining the expression of genes regulated by wild-type MLL. Since several members of the *Hoxa*, *Hoxb* and *Hoxc* gene clusters are targets of wild-type MLL (Yu *et al.*, 1995; Hess *et al.*, 1997; Yagi *et al.*, 1998; Yu *et al.*, 1998; Ernst *et al.*, 2004b), we analysed the expression profile of all 39 murine *Hox* genes and the *Meis* and *Pbx* Hox co-factors in the constitutive (c-ME1) and conditional (TRE-ME2, TRE-ME3 and TRE-ME6) MLL-ENL immortalised cell lines by Quantitative-PCR. Global gene expression profiling of the cell lines was also performed by Affymetrix microarray analysis in order to identify other non-*Hox* targets of MLL-ENL.

6.1 MLL-ENL immortalised cell lines express a specific pattern of *Hoxa* genes.

In a collaboration with Prof. Terry Lappin (Queen's University, Belfast), we analysed the global *Hox* gene expression profile of the MLL-ENL immortalised cell lines by Q-PCR. The murine *Hox* genes were cloned and standard curves were generated for 38 of the 39 genes and for the Hox co-factors *Meis-1* and *Pbx-1*. These curves were used to convert Q-PCR data, which had been normalised to 18S ribosomal RNA expression, into *Hox* gene copy number (work of D. Grier, G. McGonigle, A. Thompson and T. Lappin, Queen's University, Belfast). The expression levels of each *Hox* gene could then be compared with other *Hox* genes expressed by the same cell line and with *Hox* gene expression in other cell lines (Horton *et al.*, 2005).

All of the MLL-ENL immortalised cell lines expressed a broadly similar pattern of *Hoxa* cluster genes (Figure 6.1) (Horton *et al*, 2005). TRE-ME2 and c-ME1 expressed similar levels of the *Hoxa* cluster genes while TRE-ME3 and TRE-ME6 expressed slightly lower levels. The 5' *Hoxa* genes (excluding *Hoxa13* which was

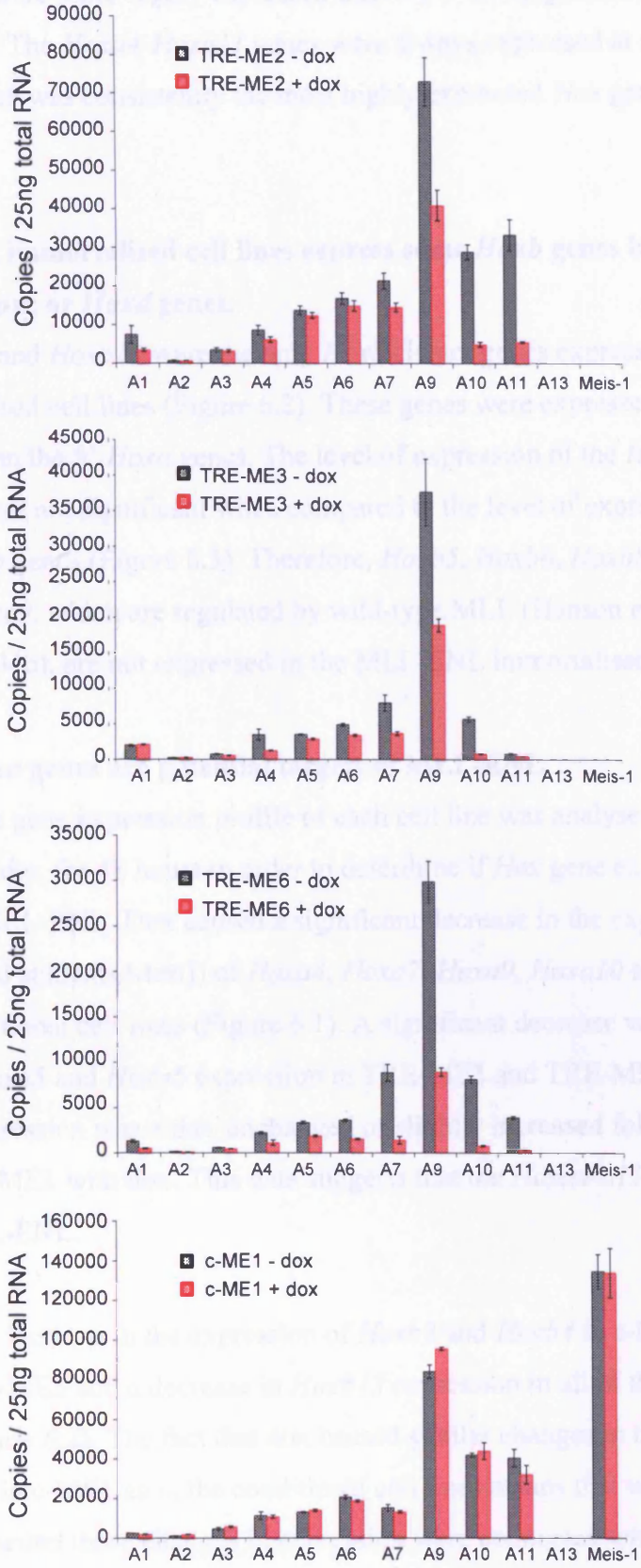

Figure 6.1 MLL-ENL cell lines express a *Hoxa* code. The graphs show the number of copies of each *Hoxa* gene per 25 ng total RNA following culture of each line in the presence (red columns) or absence (black columns) of dox for 48 hours. The mean and SD of triplicate measurements are shown.

not expressed) were more highly expressed than the 3' *Hoxa* genes in all of the lines examined. The *Hoxa4-Hoxa11* genes were always expressed at significant levels and *Hoxa9* was consistently the most highly expressed *Hox* gene (Figure 6.1).

6.2 MLL-ENL immortalised cell lines express some *Hoxb* genes but do not express any *Hoxc* or *Hoxd* genes.

Hoxb3, *Hoxb4* and *Hoxb13* were the only *Hoxb* cluster genes expressed in MLL-ENL immortalised cell lines (Figure 6.2). These genes were expressed at much lower levels than the 5' *Hoxa* genes. The level of expression of the *Hoxc* and *Hoxd* cluster genes was not significant when compared to the level of expression of the *Hoxa* and *Hoxb* genes (Figure 6.3). Therefore, *Hoxb5*, *Hoxb6*, *Hoxb8*, *Hoxc6*, *Hoxc8* and *Hoxc9*, which are regulated by wild-type MLL (Hanson *et al.*, 1999; Ernst *et al.*, 2004b), are not expressed in the MLL-ENL immortalised cell lines.

6.3 The 5' *Hoxa* genes are potential targets of MLL-ENL.

The global *Hox* gene expression profile of each cell line was analysed after treatment with dox for 48 hours in order to determine if *Hox* gene expression was regulated by MLL-ENL. Dox caused a significant decrease in the expression ($p < 0.05$ [two-tailed students t-test]) of *Hoxa4*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Hoxa11* in all of the conditional cell lines (Figure 6.1). A significant decrease was also observed in *Hoxa5* and *Hoxa6* expression in TRE-ME3 and TRE-ME6. In contrast, *Hoxa* gene expression was either unchanged or slightly increased following treatment of c-ME1 with dox. This data suggests that the *Hoxa4-a11* genes may be targets of MLL-ENL.

Dox caused an increase in the expression of *Hoxb3* and *Hoxb4* in c-ME1, TRE-ME2 and TRE-ME3 and a decrease in *Hoxb13* expression in all of the lines examined (Figure 6.2). The fact that dox caused similar changes in the expression of these genes in c-ME1 as in the conditional cell lines means that we were unable to establish whether these changes in expression were connected with the loss of MLL-ENL expression. It is known that dox alters the kinetics of the cell cycle. For example, c-ME1 proliferates at a slightly slower rate when cultured in the presence of dox (Figure 5.6). There is also evidence that the expression of certain *Hox* genes

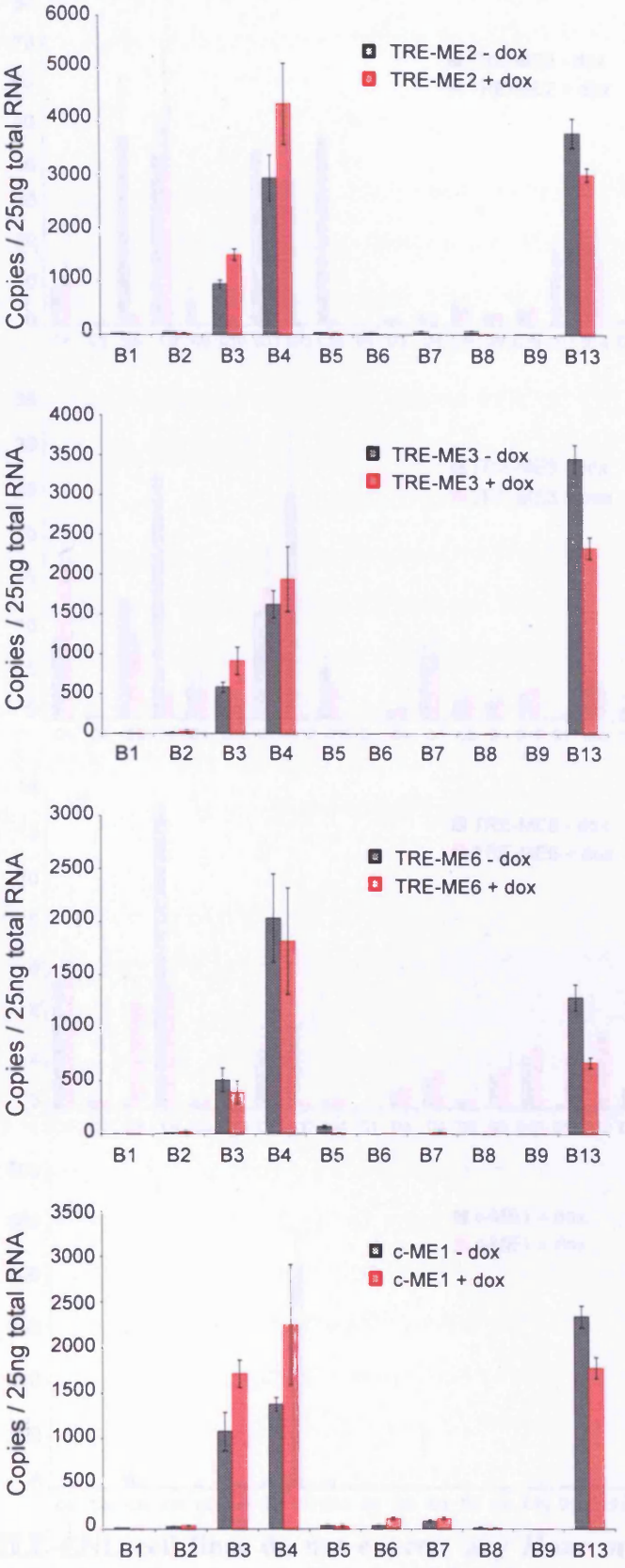

Figure 6.2 MLL-ENL cell lines express some *Hoxb* genes. The graphs show the number of copies of each *Hoxb* gene per 25 ng total RNA following culture of each cell line in the presence (red columns) or absence (black columns) of dox for 48 hours. The mean and SD of triplicate measurements are shown.

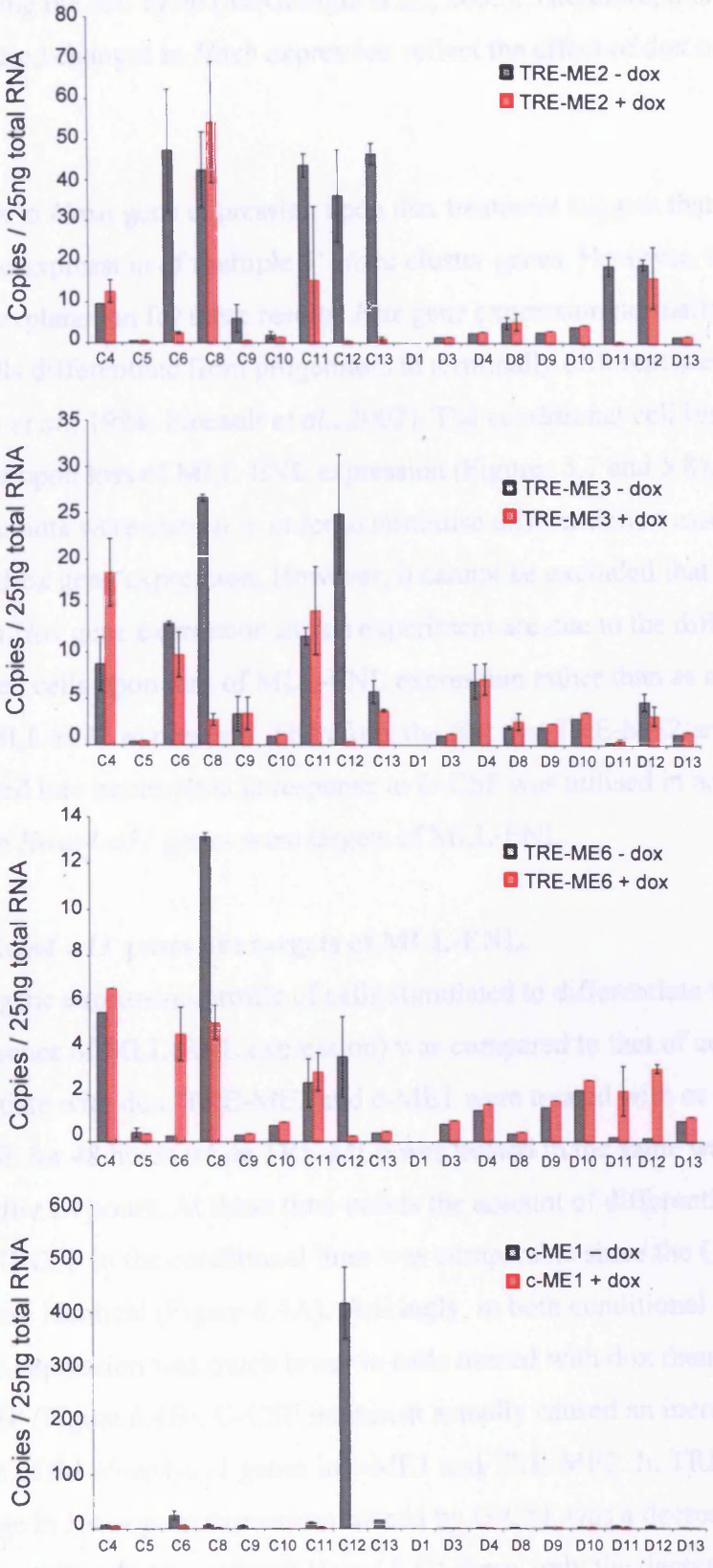

Figure 6.3 MLL-ENL cell lines do not express any *Hoxc* or *Hoxd* cluster genes. The graphs show the number of copies of the *Hoxc* and *Hoxd* genes per 25 ng total RNA following culture of each cell line in the presence (red columns) or absence (black columns) of dox for 48 hours. The mean and SD of triplicate measurements are shown.

changes during the cell cycle (McGonigle *et al.*, 2005). Therefore, it is possible that the dox induced changes in *Hoxb* expression reflect the effect of dox on slowing the cell cycle.

The changes in *Hoxa* gene expression upon dox treatment suggest that MLL-ENL regulates the expression of multiple 5' *Hoxa* cluster genes. However, there is an alternative explanation for these results. *Hox* gene expression normally decreases as myeloid cells differentiate from progenitors to terminally differentiated cells (Sauvageau *et al.*, 1994; Pineault *et al.*, 2002). The conditional cell lines differentiate upon loss of MLL-ENL expression (Figures 5.7 and 5.8), therefore early time-points were chosen in order to minimise differentiation associated changes in *Hox* gene expression. However, it cannot be excluded that the decreases observed in *Hox* gene expression in this experiment are due to the differentiation of immortalised cells upon loss of MLL-ENL expression rather than as a direct result of losing MLL-ENL expression. Therefore, the fact that TRE-ME2 and TRE-ME6 differentiated into neutrophils in response to G-CSF was utilised in addressing whether the *Hoxa4-a11* genes were targets of MLL-ENL.

6.4 The *Hoxa4-a11* genes are targets of MLL-ENL.

The *Hoxa* gene expression profile of cells stimulated to differentiate with G-CSF (in the presence of MLL-ENL expression) was compared to that of cells stimulated to differentiate with dox. TRE-ME2 and c-ME1 were treated with or without dox or with G-CSF for 48 hours while TRE-ME6 was treated in the same way but analysed after 24 hours. At these time-points the amount of differentiation induced by dox or G-CSF in the conditional lines was comparable since the Gr-1 expression profiles were identical (Figure 6.4A). Strikingly, in both conditional cell lines, *Hoxa* gene expression was much lower in cells treated with dox than in cells treated with G-CSF (Figure 6.4B). G-CSF treatment actually caused an increase in expression of the *Hoxa4-a11* genes in c-ME1 and TRE-ME2. In TRE-ME6, the only change in *Hoxa* gene expression caused by G-CSF was a decrease in the expression of *Hoxa7*, *Hoxa10* and *Hoxa11*. Of these, only the decrease in *Hoxa11* expression was of a similar magnitude to that caused by dox. Collectively, these data suggest that the decrease observed in expression of the *Hoxa4-a11* genes upon

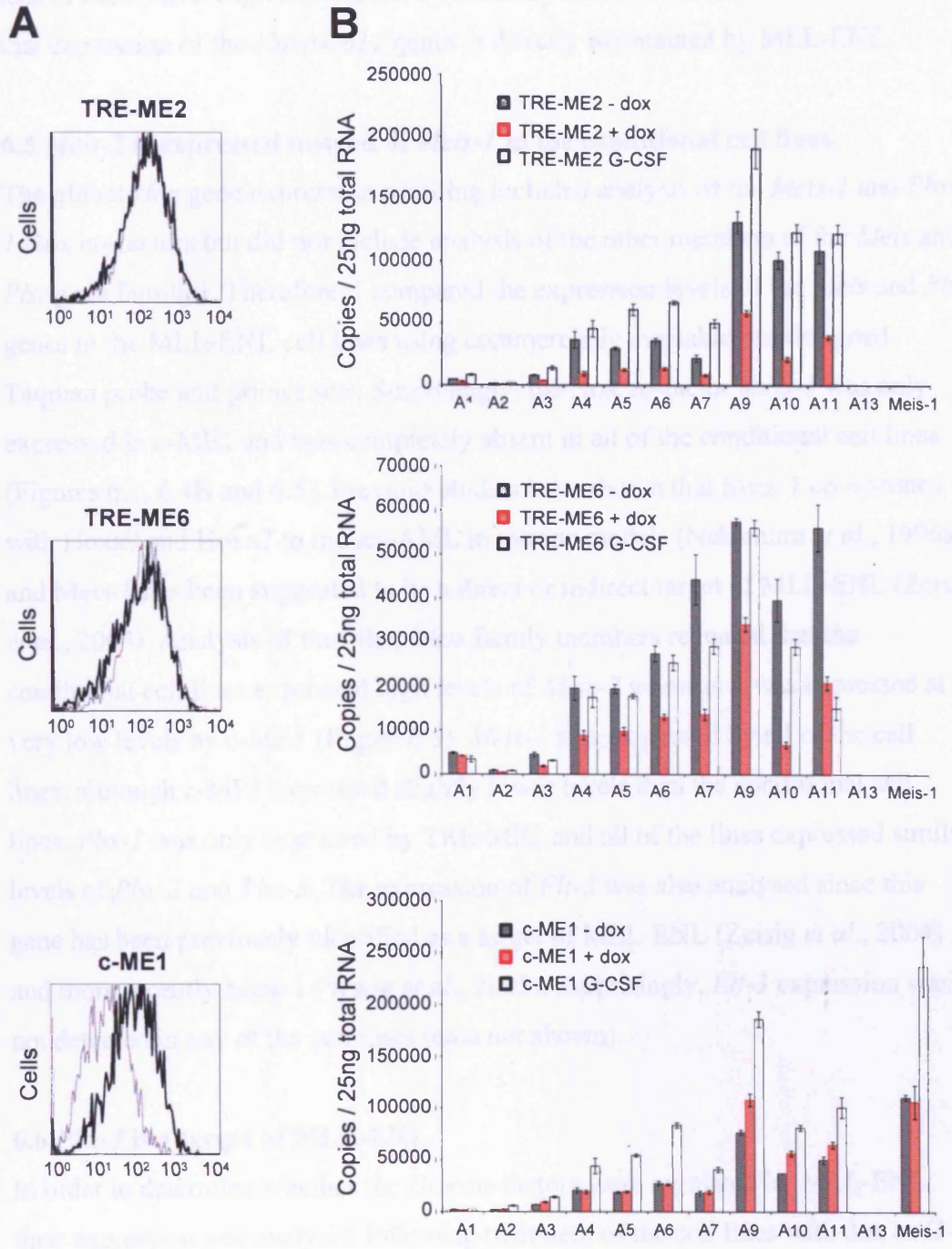

Figure 6.4 MLL-ENL maintains the expression of multiple *Hoxa* genes. The cell lines were cultured with or without dox or with G-CSF in order to compare their *Hoxa* gene expression profile by Q-PCR. **A)** Plots show the Gr-1 expression profile of c-ME1, TRE-ME2 and TRE-ME6 following culture with either dox (grey line) or G-CSF (thick black line). Cells were harvested at a time-point at which the amount of differentiation induced by dox or G-CSF was comparable. C-ME1 and TRE-ME2 were analysed after 48 hours and TRE-ME6 was analysed after 24 hours. **B)** The graphs show the number of copies of each *Hoxa* gene per 25 ng total RNA following culture of the cell lines in SCF, IL-6, IL-3 without dox (black columns), with dox (red columns), or with G-CSF (white columns). The means and SD of triplicate measurements are shown.

loss of MLL-ENL expression is not a secondary result of differentiation, but rather that expression of the *Hoxa4-a11* genes is directly maintained by MLL-ENL.

6.5 *Meis-2* is expressed instead of *Meis-1* in the conditional cell lines.

The global *Hox* gene expression profiling included analysis of the *Meis-1* and *Pbx-1* Hox co-factors but did not include analysis of the other members of the *Meis* and *Pbx* gene families. Therefore, I compared the expression levels of the *Meis* and *Pbx* genes in the MLL-ENL cell lines using commercially available pre-designed Taqman probe and primer sets. Surprisingly, the Hox co-factor *Meis-1* was only expressed in c-ME1 and was completely absent in all of the conditional cell lines (Figures 6.1, 6.4B and 6.5). Previous studies have shown that Meis-1 co-operates with Hoxa9 and Hoxa7 to induce AML in murine models (Nakamura *et al.*, 1996a) and Meis-1 has been suggested to be a direct or indirect target of MLL-ENL (Zeisig *et al.*, 2004). Analysis of the other *Meis* family members revealed that the conditional cell lines expressed high levels of *Meis-2* whereas it was expressed at very low levels by c-ME1 (Figure 6.5). *Meis-3* was expressed by all of the cell lines, although c-ME1 expressed slightly lower levels than the conditional cell lines. *Pbx-1* was only expressed by TRE-ME2 and all of the lines expressed similar levels of *Pbx-2* and *Pbx-3.* The expression of *Flt-3* was also analysed since this gene has been previously identified as a target of MLL-ENL (Zeisig *et al.*, 2004) and more recently Meis-1 (Wang *et al.*, 2005). Surprisingly, *Flt-3* expression was not detected in any of the cell lines (data not shown).

6.6 *Pbx-3* is a target of MLL-ENL.

In order to determine whether the Hox co-factors were regulated by MLL-ENL, their expression was analysed following treatment of the cell lines with dox or G-CSF for the same amount of time as that in the previous analysis of *Hoxa* gene expression (Figure 6.4). Dox had no significant effect on *Meis-2* expression (Figure 6.5). Although *Meis-3* expression decreased in the conditional cell lines in response to dox, G-CSF caused similar if not greater decreases. These changes are therefore likely to be due to differentiation of the cells. Dox caused an increase in the expression of *Pbx-2* in all of the cell lines, whereas expression was unchanged by G-CSF. Since dox caused a similar change in *Pbx-2* expression in c-ME1 as in the conditional cell lines, we were unable to determine if this gene was regulated by

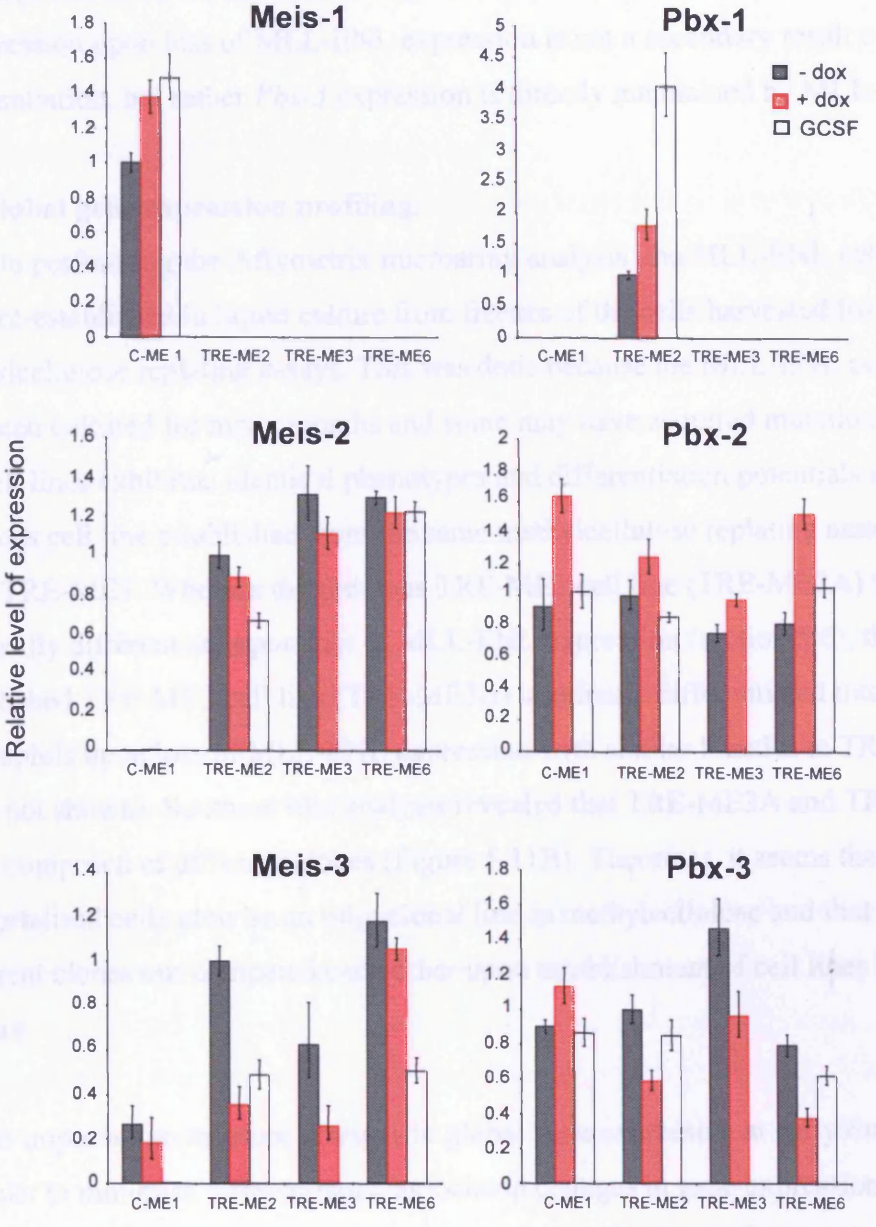

Figure 6.5 MLL-ENL maintains the expression of *Pbx-3* but not other *Hox* co-factors. The graphs show the relative level of expression of the *Meis* and *Pbx* Hox co-factor genes following culture of the cell lines in SCF, IL-6 and IL-3 without dox (black columns), with dox (red columns) or with G-CSF (white columns). Cells were analysed at time-points identical to that used in the analysis of *Hoxa* gene expression (see Figure 6.4). TRE-ME3 was not analysed after culture in G-CSF since the cells died. Q-PCR was performed using commercially available pre-designed Taqman probe and primer sets and data was normalised to 18S ribosomal RNA. The mean and SD of quadruplicate measurements are shown.

MLL-ENL. Dox caused a decrease in *Pbx-3* expression in all of the conditional cell lines. However, *Pbx-3* expression increased in c-ME1 in response to dox and was unchanged in all of the cell lines in response to G-CSF. Hence, the decrease in *Pbx-3* expression upon loss of MLL-ENL expression is not a secondary result of differentiation, but rather *Pbx-3* expression is directly maintained by MLL-ENL.

6.7 Global gene expression profiling.

Prior to performing the Affymetrix microarray analysis, the MLL-ENL cell lines were re-established in liquid culture from freezes of the cells harvested from the methylcellulose replating assays. This was done because the MLL-ENL cell lines had been cultured for many months and some may have acquired mutations. All of the cell lines exhibited identical phenotypes and differentiation potentials as the previous cell line established from the same methylcellulose replating assay apart from TRE-ME3. Whereas the previous TRE-ME3 cell line (TRE-ME3A) failed to terminally differentiate upon loss of MLL-ENL expression (section 5.6), the newly established TRE-ME3 cell line (TRE-ME3B) terminally differentiated into neutrophils upon loss of MLL-ENL expression with similar kinetics to TRE-ME2 (data not shown). Southern blot analysis revealed that TRE-ME3A and TRE-ME3B were composed of different clones (Figure 5.11B). Therefore, it seems that the immortalised cells grew as an oligoclonal line in methylcellulose and that the different clones out-competed each other upon establishment of cell lines in liquid culture.

It was important to measure changes in global gene expression at early time-points in order to minimise differentiation associated changes in gene expression and increase the chances of identifying primary targets of MLL-ENL. In order to determine a suitable time-point for the experiment, the expression of the MLL-ENL target genes *Hoxa7* and *Hoxa9* were analysed by Q-PCR using commercially available probe and primer sets. *Hox* gene expression was analysed following treatment of the MLL-ENL cell lines with or without dox for 24 and 48 hours. TRE-ME6 was only analysed after 24 hours since this cell line differentiates more rapidly than the others upon loss of MLL-ENL expression. Although a significant decrease in *Hox* gene expression was observed after 24 hours in TRE-ME2 and TRE-ME3(B) following dox treatment ($p < 0.07$ [two-tailed students t-test]), the

decrease was not as significant as that observed after 48 hours ($p < 1.4 \times 10^{-5}$ [two-tailed students t-test], Figure 6.6). Therefore, we decided to analyse the gene expression profiles of TRE-ME2, TRE-ME3(B) and c-ME1 after 48 hours and TRE-ME6 after 24 hours treatment with or without dox.

6.8 Identification of MLL-ENL target genes.

RNA was prepared from each cell line following treatment with or without dox and hybridised to Affymetrix murine 430 2.0 oligonucleotide microarrays representing more than 39,000 transcripts based on the mouse UniGene cluster (build 107). The three conditional cell lines were grouped as replicates and pairwise comparisons of these samples treated with and without dox were performed to identify differentially expressed genes. The data was filtered by selecting for genes which were present and whose expression level changed more than 1.3-fold upon treatment with dox (section 2.25). A Welch t-test was then used to identify genes which were changed significantly in the conditional cell lines upon dox treatment. The expression of the genes which were changed significantly in the conditional cell lines upon dox treatment was then examined in the constitutive cell line. This comparison was made in order to discriminate between changes in gene expression connected with loss of MLL-ENL expression versus changes in gene expression due to non-specific effects of dox. Fifty-seven percent of the genes which were changed in the conditional cell lines showed a concordant change in expression in the constitutive cell line upon dox treatment (Tables A.1 and A.2). Since only one constitutive cell line was analysed and replicates were not performed, it was not possible to determine whether the concordant changes in gene expression in the constitutive cell line were reproducible. Therefore, genes such as *Ikrak1bp* and *Sap30* (Table A.1), which have been previously identified as up-regulated in patient samples with 11q23 translocations, should be analysed in more detail by Q-PCR. Genes which did not show a concordant change in expression in the constitutive cell line upon dox treatment are potentially targets of MLL-ENL or its downstream effectors and are the focus of the remainder of this chapter.

6.9 Genes down-regulated following loss of MLL-ENL expression.

In total, 30 genes were down-regulated in the three conditional cell lines upon loss of MLL-ENL expression (Table 6.1). These included the transcription factors

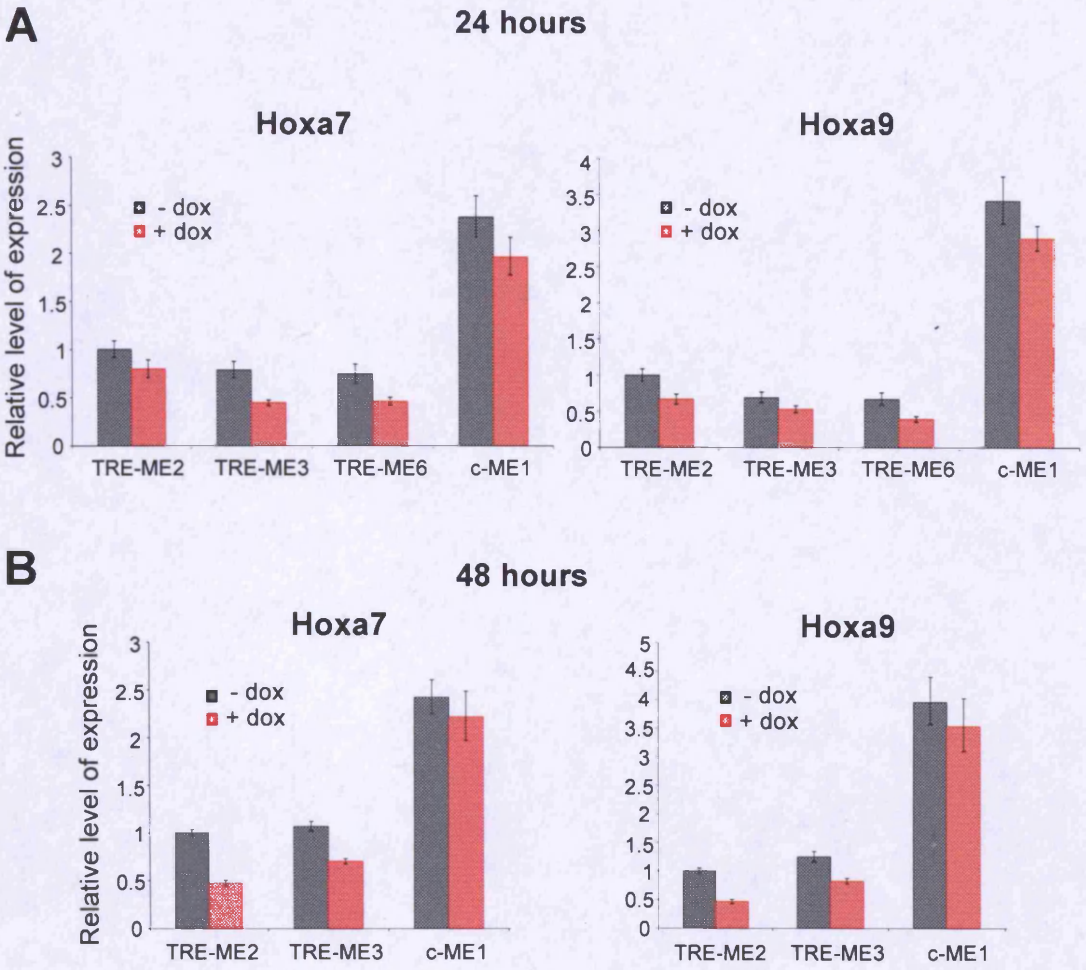

Figure 6.6 Identification of a suitable time-point for global gene expression profiling. The graphs show the relative level of *Hoxa7* and *Hoxa9* expression following culture of each cell line with (red columns) or without (black columns) dox for **A)** 24 or **B)** 48 hours. Q-PCR was performed using commercially available pre-designed Taqman probe and primer sets and data was normalised to 18S ribosomal RNA. TRE-ME6 was not analysed after 48 hours since this cell line differentiates more rapidly than the other conditional cell lines upon loss of MLL-ENL expression. The mean and SD of quadruplicate measurements are shown.

Hoxa9 and *Sdccag33*. The differential expression of *Hoxa9* confirmed the Q-PCR analysis (Figure 6.4B) and validated the experiment. *Sdccag33* (also known as *Mtsh1*) is the murine orthologue of the *Drosophila Teashirt* (*Tsh*) gene. Like the HOM-C proteins of Drosophila, Tsh is a transcription factor which determines segment identity during development (Alexandre *et al.*, 1996). Interestingly, the Tsh and HOM-C transcription factors share some common target genes (Alexandre *et al.*, 1996). Therefore, it will be interesting to determine if Mtsh1 and the Hoxa proteins share common targets in haematopoietic cells and whether these genes co-operate to induce leukaemogenesis.

Other genes involved in transcription that were down-regulated upon loss of MLL-ENL expression include *Elp3* and *Mettl2*. Elp3 is a component of the yeast Elongator complex which possesses histone acetyltransferase activity (Winkler *et al.*, 2002). Elongator associates with RNA polymerase II and promotes transcriptional elongation by acetylating histones and thereby maintaining an open chromatin structure. Mettl2 may also promote transcriptional initiation or elongation if it possesses histone methyltransferase activity.

The *Aldehyde dehydrogenase 1, family member L2* (*Aldh1l2*) gene showed the greatest fold decrease upon loss of MLL-ENL expression (Table 6.1). Aldehyde dehydrogenase is an enzyme that oxidises intracellular aldehydes and is thought to confer resistance to cyclophosphamide (Magni *et al.*, 1996). Interestingly, *Aldh1* has been previously identified as a direct target of Hoxa9 in myeloid cell lines (Dorsam *et al.*, 2004). The *Pim-1* oncogene is also a target of Hoxa9 (Dorsam *et al.*, 2004). We found that the close family member *Pim-2* was down-regulated upon loss of MLL-ENL expression. Pim-2 confers protection from apoptosis in response to growth factor withdrawal (Fox *et al.*, 2003) and it is over-expressed in several types of cancer in humans including leukaemias (Amson *et al.*, 1989). Hence, *Pim-2* is an attractive candidate target gene to explore in more detail. Other interesting target genes include *Mtss1* which is an actin-binding protein that regulates the actin cytoskeleton (Gonzalez-Quevedo *et al.*, 2005) and also plays a role in transcription. It is a member of the Sonic Hedgehog signalling pathway that potentiates Gli-dependent transcription during development and carcinogenesis (Callahan *et al.*, 2004).

Table 6.1 Genes down-regulated upon loss of MLL-ENL expression.

Gene	P-value	Relative expression (RE)					Product
		Average RE	TRE-ME2	TRE-ME3	TRE-ME6	c-ME1	
Aldh1l2	0.0142	0.24	0.12	0.20	0.40	1.59	Aldehyde dehydrogenase 1 family, member L2
Magi1	0.0102	0.32	0.29	0.43	0.24	1.69	Membrane associated guanylate kinase interacting protein-like 1
Hig1	0.0268	0.40	0.23	0.29	0.69	0.83	Hypoxia induced gene 1
Sdccag33	0.00614	0.46	0.45	0.30	0.63	0.97	Serologically defined colon cancer antigen 33
4921530L18Rik	0.0115	0.53	0.43	0.43	0.72	0.87	Unknown EST
Hoxa9	0.0291	0.55	0.67	0.41	0.56	1.08	Homeobox transcription factor
Mtss1	0.0424	0.55	0.40	0.42	0.83	0.94	Metastasis suppressor 1
5730405O12Rik	0.0256	0.58	0.50	0.65	0.58	1.75	Unclassifiable
4833442J19Rik	0.0487	0.58	0.60	0.63	0.50	1.19	Hypothetical protein LOC320204
Senp8	0.0381	0.6	0.73	0.5	0.57	1	SUMO / senthrin specific protease 8
Asf1a	0.0132	0.61	0.63	0.56	0.63	0.86	Anti-silencing function 1 homolog A (S.cerevisiae)

1700037C18Rik	0.0253	0.61	0.68	0.52	0.64	1.01	Hypothetical protein LOC73261
Elp3	0.0487	0.65	0.54	0.68	0.73	0.92	Elongation protein 3 homolog (S.cerevisiae)
HelB	0.00842	0.65	0.57	0.56	0.81	0.88	Helicase (DNA) B
Pim2	0.0207	0.65	0.66	0.55	0.74	1.65	Proviral integration site 2
Anp32b	0.0345	0.66	0.49	0.65	0.85	1.25	Acidic nuclear phosphoprotein 32, family member B
Amotl1	0.0482	0.68	0.7	0.68	0.67	1.49	Angiomotin-like 1
Ierepo4	0.045	0.70	0.78	0.56	0.75	1.18	Immediate early response, erythropoietin 4
9530078B04Rik	0.0399	0.70	0.85	0.56	0.70	6.07	Unclassifiable
Taok1	0.0409	0.71	0.76	0.53	0.83	1.20	Thousand and one amino acid protein kinase 1
2510005D08Rik	0.00429	0.71	0.79	0.63	0.71	1.25	Hypothetical protein LOC68043
Cdc27	0.0453	0.73	0.61	0.78	0.80	1.44	Cell division cycle 27 homolog (S.cerevisiae)
4933432P15Rik	0.0311	0.73	0.63	0.72	0.84	0.86	Similar to GRAF gene
Ap3s2	0.0212	0.74	0.65	0.91	0.67	1.79	Adaptor related protein complex 3, sigma 2 subunit
Timm8a	0.0237	0.74	0.80	0.76	0.65	1.14	Translocase of inner mitochondrial membrane 8 homolog a (yeast)

Paip1	0.0293	0.75	0.79	0.69	0.76	1.18	Polyadenylate binding protein-interacting protein 1
A730091E23Rik	0.0463	0.75	0.71	0.59	0.95	1.23	Unknown EST
Mettl2	0.0461	0.75	0.93	0.66	0.67	1.03	Methyltransferase-like2
Alg12	0.00618	0.77	0.77	0.75	0.78	0.88	Asparagine-linked glycosylation 12 homolog (yeast α-1,6-mannosyltransferase)
Nomo1	0.0233	0.77	0.73	0.81	0.76	1.14	Nodal modulator 1

Relative Expression is the level of gene expression in the presence of dox relative to that in the absence of dox. Genes in red are over-expressed in patients with 11q23 translocations compared to other leukaemias (Armstrong *et al.*, 2002, Ross *et al.*, 2004, Andersson *et al.*, 2005, Kohlmann *et al.*, 2005).

Several genes required for cell cycle progression were down-regulated upon loss of MLL-ENL expression (Table 6.2). These included *Anp32b*, a *Cdc27* homologue, an *Asf1* homologue and *Hel(B)*. Anp32b (also known as Pal31), is a nuclear phosphoprotein required for S phase progression (Sun *et al.*, 2001). Cdc27 is a component of the yeast DNA polymerase δ complex. It is essential for DNA replication since it recruits proliferating cell nuclear antigen (PCNA) to the polymerase complex (Bermudez *et al.*, 2002). Asf1 is essential for cell cycle progression in yeast since it assembles chromatin following DNA replication (Tyler *et al.*, 1999) and Hel(B) is required to unwind DNA prior to replication (Matsumoto *et al.*, 1995).

Table 6.2 Classification of genes down-regulated upon loss of MLL-ENL expression according to function.

Function	Gene
Cell signalling	Pim2 Magi1 Taok1
Cell cycle	Anp32b Asf1a Cdc27 HelB
Cytoskeleton regulation	Mtss1
Protein targeting / transport	Ap3s2 Senp8 Timm8a
Transcription regulation	Elp3 Hoxa9 Mettl2 Sdccag33
Translation regulation	Paip1

6.10 Genes up-regulated following loss of MLL-ENL expression.

Thirty-six genes were up-regulated in the three conditional cell lines upon loss of MLL-ENL expression (Table 6.3). These included several genes whose products

Table 6.3 Genes up-regulated upon loss of MLL-ENL expression.

Gene	P-value	Relative Expression (RE)					Product
		Average RE	TRE-ME2	TRE-ME3	TRE-ME6	c-ME1	
Camp	0.0407	14.46	25.00	12.5	5.88	1.04	Cathelicidin antimicrobial peptide
Pglyrp	0.0298	10.53	20.00	9.09	2.50	0.85	Peptidogylcan recognition protein
Ngp	0.00694	6.00	5.88	9.09	3.03	0.50	Neutrophil granule protein
Ceacam10	0.013	5.49	7.14	5.88	3.45	0.09	CEA-related cell adhesion molecule 10
Pram1	0.0288	3.54	2.94	4.35	3.33	0.68	PML-RARα regulated adaptor molecule 1 (PRAM-1) protein
1190003K14Rik	0.0193	2.76	4.35	1.67	2.27	0.46	Unclassified
Ddb2	0.00158	2.41	2.27	2.63	2.33	0.63	Damage specific DNA binding protein 2
Prkd2	0.014	2.25	2.5	1.30	2.94	0.71	Protein kinase D2
Ceacam1	0.0384	2.12	2.94	1.82	1.61	0.41	CEA-related cell adhesion molecule 1
A130086G11Rik	0.047	2.03	1.75	2.38	1.96	1.11	Unknown EST
Hook2	0.042	1.92	2.17	2.13	1.45	0.14	Hook homolog 2 (Drosophila)

Wdr37	0.0282	1.84	1.72	2.17	1.64	0.28	WD repeat domain 37
Cnn2	0.0376	1.79	2.13	1.69	1.56	0.49	Calponin 2
Cybasc3	0.0187	1.78	1.41	2.08	1.85	0.95	Cytochrome b, ascorbate dependent 3
Mt1	0.0125	1.70	1.35	1.61	2.13	0.39	Metallothionein 1
Arhgap19	0.012	1.58	1.59	1.64	1.52	0.38	Rho GTPase activating protein 19
1190002H09Rik	0.0188	1.58	1.56	1.61	1.56	0.39	Hypothetical protein LOC68857
Nfatc1	0.0362	1.54	1.37	1.18	2.08	0.56	Nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1
6230416A05Rik	0.037	1.54	1.75	1.45	1.41	0.45	Hypothetical protein LOC76137
Map4k2	0.0236	1.53	1.69	1.35	1.54	0.37	Mitogen activated protein kinase kinase kinase kinase 2
C030046I01Rik	0.0483	1.52	1.67	1.22	1.67	0.81	Hypothetical protein LOC109284
Bri3bp	0.0293	1.49	1.96	1.19	1.33	1.11	Bri 3 binding protein
Apg7l	0.0422	1.45	1.82	1.19	1.33	0.16	Autophagy 7-like (S.cerevisiae)
Fln29	0.0452	1.43	1.52	1.33	1.45	0.66	FLN29 gene product
Actn4	0.0152	1.42	1.64	1.27	1.35	0.23	Actinin alpha 4

Aatk	0.0159	1.41	1.39	1.33	1.52	0.89	Apoptosis-associated tyrosine kinase
A630020E03Rik	0.0339	1.40	1.27	1.39	1.54	0.49	Unknown EST
Inpp5f	0.00896	1.37	1.35	1.3	1.47	0.72	Inositol polyphosphate-5-phosphatase F
Ypel5	0.0339	1.37	1.39	1.61	1.11	0.62	Yippee-like 5 (Drosophila)
Atrx	0.0234	1.35	1.49	1.37	1.19	0.29	Alpha thalassemia / mental retardation syndrome X linked homolog (human)
Gyg1	0.0256	1.33	1.16	1.56	1.27	0.48	Glycogenin 1
D19Wsu12e	0.0168	1.33	1.32	1.39	1.27	0.67	Similar to human KIAA1815 protein
Slc25a30	0.0417	1.33	1.64	1.06	1.28	0.99	Solute family carrier 25, member 30
Crlf3	0.047	1.32	1.35	1.27	1.33	0.62	Cytokine receptor-like factor 3
Sri	0.012	1.31	1.28	1.27	1.39	0.49	Sorcin

Relative Expression is the level of gene expression in the presence of dox relative to that in the absence of dox. Genes in red are over-expressed in patients with 11q23 translocations compared to other leukaemias (Armstrong *et al.*, 2002, Ross *et al.*, 2004, Andersson *et al.*, 2005, Kohlmann *et al.*, 2005).

are involved in myeloid cell differentiation and function such as the secondary granule protein Ngp, Pram-1 which is required for mature neutrophil function (Clemens *et al.*, 2004) and Camp and Pglyrp which are involved in the recognition and destruction of pathogens (Table 6.4). The differential expression of such genes was expected since the cells differentiate upon loss of MLL-ENL expression.

The genes *Nfatc1* and *Atrx*, whose products are involved in transcriptional regulation, were also up-regulated following loss of MLL-ENL expression. Nfatc1 is a transcription factor which regulates the expression of cytokine genes in activated T lymphocytes (Zhou *et al.*, 2002) and Atrx is a SWI / SNF chromatin remodelling protein which is mutated in X-linked mental retardation syndromes (Park *et al.*, 2004). Interestingly, inactivating mutations of Atrx are found in patients with acquired alpha thalassemia associated with myelodysplastic syndrome (Steensma *et al.*, 2005). Therefore, it is feasible that transcriptional repression of this gene by MLL-ENL contributes to leukaemogenesis.

Other genes that were up-regulated following loss of MLL-ENL expression include *Actn4* and *Cnn2*, whose products are both actin binding proteins. Previous studies have suggested that Actn4 is a tumour suppressor since it is mutated in some human lung carcinomas and over-expression of the wild type gene inhibits tumour cell growth *in vivo* (Menez *et al.*, 2004). Furthermore, over-expression of Actn4 in malignant neuroblastoma cells results in decreased anchorage independent growth and loss of tumorigenecity *in vivo* (Nikolopoulos *et al.*, 2000). Cnn2 (also known as h2 calponin) is another actin binding protein which is thought to inhibit proliferation by inhibiting cytokinesis in smooth muscle cells (Hossain *et al.*, 2003).

Of particular interest was the fact that several genes whose products are required for apoptosis such as *Aatk*, *Ddb2* and *Bri3 binding protein* were up-regulated following loss of MLL-ENL expression. Aatk is required for growth arrest and apoptosis of terminally differentiated myeloid cells (Gaozza *et al.*, 1997). Ddb2 is a tumour suppressor which is mutated in patients with Xeroderma pigmentosam group E. Patients with this disease are susceptible to UV light-induced skin cancers (Itoh *et al.*, 2001), since Ddb2 is required to induce p53 mediated apoptosis in response to UV irradiation (Itoh *et al.*, 2004). Bri3 is required for apoptosis in response to

tumour necrosis factor (TNF) (Wu *et al.*, 2003). Thus, it is likely that the function of Bri3 binding protein will be to regulate this process. It will be interesting to determine whether the repression of these genes by MLL-ENL contributes to leukaemogenesis.

Table 6.4 Classification of genes up-regulated upon loss of MLL-ENL expression according to function.

Function	Gene
Cell signalling	Arhgap19 Inpp5f Map4k2 Prdk2
Cytoskeleton regulation	Actn4 Cnn2
Myeloid immune function	Camp Ceacam1 Cecam10 Crlf3 Ngp Pglyrp Pram1
DNA repair and apoptosis	Aatk Bri3bp Ddb2
Transcription regulation	Atrx Nfatc1

Discussion

Global *Hox* gene expression profiling of MLL-ENL immortalised cell lines revealed that these cells expressed a *Hoxa* code which consisted of *Hoxa4*, *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Hoxa11*. Interestingly, the absolute levels of *Hoxa* gene expression broadly correlated with the rate of proliferation of each conditional cell line. For example, TRE-ME2 proliferated at a faster rate than TRE-ME3 or TRE-ME6 and expressed the highest levels of *Hoxa* genes. Furthermore, TRE-ME3 proliferated at a slower rate than TRE-ME2 and TRE-ME6 and expressed the lowest overall level of *Hoxa* genes. The absolute levels of *Hoxa* gene expression in the conditional cell lines increased over time (compare Figures 6.1 and 6.4B). However, a similar increase in *Hoxa* gene expression was not observed for the constitutive cell line. It is possible that *Hox* genes confer a selection advantage to the cells, such that over time, the cells express higher levels of these genes. This selection advantage may not be as strong for the constitutive cell line since this line expresses *Meis-1* which has been shown to provide a clonal selection advantage to myeloid cells immortalised by Hoxa9 (Wang *et al.*, 2005).

Expression of *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Hoxa11* in MLL-ENL immortalised cell lines has been previously reported (Ayton and Cleary, 2003; So *et al.*, 2003b). However, *Hoxa* gene expression is not restricted to MLL-ENL cell lines, since some *Hoxa* genes are also expressed by myeloid cell lines immortalised by E2A-HLF (So *et al.*, 2004). Therefore, it is possible that these cells express particular *Hoxa* genes because of a differentiation block imposed by MLL-ENL and that this depends on the *Hox* gene expression of the original immortalised myeloid progenitors. In order to distinguish between this explanation and the possibility that MLL-ENL was directly responsible for maintaining *Hox* gene expression in these lines, *Hox* gene expression was analysed following treatment of the MLL-ENL cell lines with dox and with G-CSF. A significant decrease in *Hoxa* gene expression was observed upon loss of MLL-ENL expression. This was not a secondary result of differentiation, since treatment of cells at an identical stage of differentiation induced by G-CSF, did not result in an equivalent decrease in expression. Therefore, MLL-ENL directly maintains the expression of the *Hoxa4-a11* genes. This result is supported by the finding that MLL-FKBP and MLL-AF10 bind to regulatory regions of the *Hoxa7* and *Hoxa9* promoters in transformed cells (Martin

et al., 2003; Okada *et al.*, 2005). The increase in *Hoxa* gene expression in TRE-ME2 and c-ME1 in response to G-CSF was not expected since *HOX* gene expression normally decreases as myeloid cells differentiate (Sauvageau *et al.*, 1994; Pineault *et al.*, 2002). These results suggest that MLL-ENL can maintain *Hoxa* gene expression even when the cells are differentiating. Further analysis of *Hoxa* gene expression at later stages of G-CSF induced differentiation in these cells is required to confirm this hypothesis.

Like MLL-ENL immortalised cell lines, Hoxa9 immortalised cell lines continuously proliferate in response to GM-CSF but terminally differentiate in response to G-CSF (Calvo *et al.*, 2000). The authors suggest that Hoxa9 might block GM-CSF induced differentiation by suppressing the expression of transcription factors essential for GM-CSF signalling such as STAT5 (Calvo *et al.*, 2000). MLL-ENL immortalised cells might retain the ability to differentiate in response to G-CSF if factors required for G-CSF signalling, such as STAT3 (Shimozaki *et al.*, 1997), are not altered by the over-expression of *Hoxa* genes. Alternatively, G-CSF signalling may overcome the differentiation block imposed by Hoxa proteins by modulating the expression of genes required by the Hox proteins to exert their effect.

The *Hox* code expressed in MLL-ENL immortalised cell lines is very similar to that expressed in bone marrow cells from leukaemic MLL-AF9 knock-in mice (Kumar *et al.*, 2004). Bone marrow cells from MLL-AF9 knock-in leukaemic mice expressed higher levels of *Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9*, *Hoxa10* and *Meis-1* than cells from wild-type or pre-leukaemic mice. Given the strong homology between AF9 and ENL, it is easy to envisage that MLL-AF9 and MLL-ENL de-regulate a common set of target genes. These murine models recapitulate the *HOX* gene deregulation observed in patients with *MLL* translocations. Gene expression profiling of patient material revealed that *HOXA4*, *HOXA5*, *HOXA9*, *HOXA10* and *MEIS-1* were over-expressed in both myeloid and lymphoid leukaemias with *MLL*-rearrangements (Armstrong *et al.*, 2002; Debernardi *et al.*, 2003; Ross *et al.*, 2004; Kohlmann *et al.*, 2005).

Wild type MLL maintains the expression of multiple *Hox* genes during embryogenesis including *Hoxa7*, *Hoxa9* and *Hoxa10* (Yu *et al.*, 1995; Hess *et al.*, 1997; Yagi *et al.*, 1998; Yu *et al.*, 1998; Ernst *et al.*, 2004b). Since these genes are also targets of the MLL-ENL fusion protein, a current hypothesis is that MLL-fusion proteins immortalise cells by aberrantly maintaining the expression of these genes. However, several members of the *Hoxb* and *Hoxc* gene clusters which are regulated by wild-type MLL during embryogenesis such as *Hoxb5*, *Hoxb6*, *Hoxb8* and *Hoxc8*, were not expressed in MLL-ENL immortalised cell lines. *Hoxb3*, *Hoxb4* and *Hoxb13* were expressed in MLL-ENL cell lines but significant levels of expression of members of the *Hoxc* or *Hoxd* clusters were not detected. Recent studies suggest that *Hoxb3* and *Hoxb4* are also regulated by wild-type MLL (Ernst *et al.*, 2004b). However, it was not possible to determine if these genes were regulated by MLL-ENL since dox induced similar changes in expression of these genes in both the constitutive and conditional cell lines.

It has been suggested that *Meis-1* is a target of MLL-ENL (Zeisig *et al.*, 2004). Although *Meis-1* expression is not regulated by wild-type MLL (Ernst *et al.*, 2004b), it is possible that it is regulated by MLL-ENL since the ENL moiety may confer a change in the DNA binding and transactivation specificity of MLL. The MLL-ENL fusion protein may thus be able to regulate the expression of genes which are not regulated by wild-type MLL. Alternatively, it is possible that *Meis-1* is an indirect target of MLL-ENL or it is up-regulated as a secondary event in leukaemogenesis. Our data are consistent with the latter possibility since although *Meis-1* was expressed in the constitutive cell line, it was not expressed in any of the conditional MLL-ENL cell lines. A potential mechanism which might explain why *Meis-1* was expressed in the constitutive cell line but not the conditional cell lines is that tTA may sequester transcription factors that are required for *Meis-1* expression away from the *Meis-1* promoter.

Interestingly, the conditional MLL-ENL cell lines expressed *Meis-2* instead of *Meis-1*. The Meis family consists of three members Meis-1, Meis-2 and Meis-3. Meis-2 and Meis-3 share strong homology with Meis-1, 77.2% and 69.9% respectively. Their homology is even greater within the homeodomain (96.8% identity among the three proteins) (Nakamura *et al.*, 1996b). Although *Meis-2*

expression in MLL-ENL cell lines has not been previously reported, this gene is expressed in some murine (Nakamura *et al.*, 1996b) and human myeloid leukaemias (Andersson *et al.*, 2005). Interestingly, *Meis-2* and *Meis-3* were expressed in the absence of *Meis-1* in the BXH-2 leukaemic cell lines (Nakamura *et al.*, 1996b). *Meis-2* is also expressed in the absence of *Meis-1* in WEHI-3B cells.

The Pbx and Meis Hox co-factors can form heterotrimeric DNA binding complexes with Hox proteins and are thought to increase the specificity and affinity with which Hox proteins bind to their target DNA sequences (Shen *et al*, 1996; Shen *et al.*, 1997; Shen *et al.*, 1999). *Pbx-3* was the only Hox co-factor whose expression was maintained by MLL-ENL. This finding is consistent with that of a previous study in which *Pbx-3* expression decreased upon loss of MLL-ENL function in myeloid immortalised cell lines (Zeisig *et al.*, 2004). Interestingly, *PBX-3* is over-expressed in patients with *MLL*-translocations when compared to other AML or ALL patients (Andersson *et al.*, 2005). It has been reported that in the presence of Pbx-3, Hoxa9 can interact with either Meis-1 or Meis-2 on target enhancers and that over-expression of *Hoxa9*, *Meis-1* or *Meis-2* alone can block G-CSF induced differentiation of 32Dcl3 cells (Fujino *et al.*, 2001). Hence, Meis-2 shares some of the functions of Meis-1 in myeloid progenitors. A more recent study demonstrated that Meis-1 required its Pbx interaction motif, DNA binding domain and a novel transcriptional activation domain in the C terminus to accelerate leukaemogenesis induced by *Hoxa9* over-expression (Huang *et al.*, 2005; Wang *et al.*, 2005). Since the C-terminal transactivation domain of Meis-1 is highly conserved between Meis-1, Meis-2 and Meis-3, we hypothesise that Meis-2 can substitute for Meis-1 in the conditional MLL-ENL cell lines. *MEIS-1* is frequently over-expressed in patients with *MLL*- translocations (Armstrong *et al.*, 2002; Ferrando *et al.*, 2003; Rozovskaia *et al.*, 2003; Ross *et al.*, 2004; Kohlmann *et al.*, 2005). However, it is not over-expressed in all patient samples (Drabkin *et al.*, 2002). Therefore, it will be interesting to examine if *MEIS-2* is expressed instead of *MEIS-1* in these patients.

Conflicting studies have been published on the requirement for individual *Hox* genes in MLL-fusion protein mediated leukaemogenesis. It has been shown that MLL-ENL is not able to immortalise HPCs isolated from *Hoxa9*$^{-/-}$ or *Hoxa7*$^{-/-}$ mice.

Hoxa7 and *Hoxa9* deficient mice display reduced numbers of committed progenitors (So and Cleary, 2003). However, *Hoxa9*$^{-/-}$ progenitors were susceptible to immortalisation by MLL-ENL since co-transduction of these progenitors with *MLL-ENL* and *Hoxa9* yielded immortalised cell lines (Ayton and Cleary, 2003). This study clearly demonstrated that *Hoxa7* and *Hoxa9* were required by MLL-ENL to immortalise HPCs. However, the same group demonstrated that MLL-GAS7 could immortalise *Hoxa9*$^{-/-}$ or *Hoxa7*$^{-/-}$ HPCs although with reduced efficiency (So *et al.*, 2004). Furthermore, the authors report that MLL-ENL was able to promote the *in vitro* self-renewal of *Hoxa9*$^{-/-}$ or *Hoxa7*$^{-/-}$ HPCs. This result is not in agreement with the conclusion of their previous study (Ayton and Cleary, 2003). The authors suggest that these contrasting results may be attributable to different methods of HPC isolation in the two studies.

A separate study analysed the ability of the knock-in *MLL-AF9* mutation to promote leukaemogenesis of *Hoxa9* deficient mice. *MLL-AF9*$^{+/-}$*/Hoxa9*$^{-/-}$ mice developed AML with the same penetrance and latency as *MLL-AF9*$^{+/-}$*/Hoxa9*$^{+/+}$ mice (Kumar *et al.*, 2004). However, the leukaemias of the *Hoxa9*-deficient mice were of a more immature phenotype than those of the *Hoxa9* wild-type mice. Therefore, although *Hoxa9* is not essential for MLL-AF9 induced leukaemogenesis, it does play a role in determining the phenotype of the leukaemia.

Individually *Hoxa7* and *Hoxa9* are dispensable for leukaemogenesis mediated by certain MLL-fusion proteins. It is possible that other members of the 5' *Hoxa* cluster such as *Hoxa5*, *Hoxa6* or *Hoxa10* may compensate for the lack of *Hoxa7* or *Hoxa9* expression in these models. In support of this hypothesis, a recent study demonstrated that transduction of *MLL*$^{-/-}$ embryoid bodies with *Hoxa9*, *Hoxa10* or *Hoxb4* was able to completely rescue haematopoietic colony formation (Ernst *et al.*, 2004b). Hence, these *Hox* genes share a common function in promoting haematopoietic development. Since redundancy among certain *Hox* genes exists, it is likely that therapeutic strategies designed to treat *MLL*-rearranged leukaemias which target individual *HOX* genes will not be efficacious. The identification of *HOXA* target genes and other genes regulated by MLL-ENL will probably represent a better therapeutic avenue.

Global gene expression profiling was performed using Affymetrix microarray analysis in order to identify other targets of MLL-ENL. The number of genes down-regulated upon loss of MLL-ENL expression was similar to the number of genes up-regulated upon loss of MLL-ENL expression. This suggests that MLL-ENL can repress the expression of some genes while activating or maintaining the expression of others. Although previous studies have shown than MLL-ENL can transactivate certain promoters *in vitro*, including that of *Hoxa7* (Schreiner *et al.*, 1999), it remains to be determined as to whether the genes up-regulated upon loss of MLL-ENL expression are directly repressed by MLL-ENL. The fact that the transcriptional repression domain of MLL is retained in MLL fusion proteins (Zeleznik-Le *et al.*, 1994) and the fact that ENL interacts with the transcriptional repressor human polycomb 3 (hPc3) (Garcia-Cuellar *et al.*, 2001) lends support to the hypothesis that MLL-ENL can repress the transcription of some of its target genes.

The microarray results will be confirmed by Q-PCR analysis of individual target genes. In addition, we will separate the class of genes whose change in expression is associated with differentiation by comparing the changes in gene expression of cells stimulated to differentiate with dox to that of cells stimulated to differentiate with G-CSF. Of the genes down-regulated upon loss of MLL-ENL expression, those involved in signalling pathways, the cell cycle and transcriptional regulation are probably the most interesting MLL-ENL candidate target genes. Pim-2 is of particular interest because of its ability to promote the growth and survival of haematopoietic cells in response to a variety of apoptotic stimuli (Fox *et al.*, 2003). Pim-1, Pim-2 and Pim-3 were first identified as frequent sites of retroviral insertion in lymphomas which developed following infection with the Murine Moloney Leukaemia virus (Breuer *et al.*, 1989; Mikkers *et al.*, 2002). Subsequently, it has been shown that Pim-2 and c-myc co-operate to induce lymphomas in transgenic mice (Allen *et al.*, 1997). Interestingly, a previous study demonstrated that c-myc also co-operated with MLL-ENL to transform haematopoietic progenitors and that MLL-ENL was unable to transform cells that lacked *c-myc* (Schreiner *et al.*, 2001).

Of known Hox target genes only *Aldh1*, which is a target of Hoxa9, was identified in this study. Interestingly, HSCs and LSCs exhibit a high level of Aldh1 activity

and this activity has been utilised in the isolation of these stem cell populations (Pearce *et al.*, 2005). Aldh1 confers resistance to cyclophosphamide (an alkylating agent commonly used in chemotherapeutic regimens) in haematopoietic cells (Magni *et al.*, 1996). Hence, the direct activation of *Aldh1* by Hoxa9 may contribute to a mechanism which explains the correlation between Hoxa9 over-expression and poor prognosis in AML patients (Golub *et al.*, 1999).

Several of the genes up-regulated upon loss of MLL-ENL expression, i.e. genes which are repressed by MLL-ENL, are mediators of apoptosis. Therefore, MLL-ENL may contribute to leukaemogenesis by blocking apoptosis. Indeed, MLL-AF9, MLL-ELL and MLL-ENL can block GADD34 induced apoptosis in response to gamma irradiation (Adler *et al.*, 1999). The mechanism underlying the protection from GADD34 induced apoptosis remains unclear. However, these fusion proteins have been shown to impair the transcriptional activity of p53 in response to DNA damage by reducing p53 acetylation by p300 (Wiederschain *et al.*, 2005). It is possible that MLL-ENL inhibits the function of p53 not only by disrupting its acetylation, but also by repressing the transcription of genes such as *Ddb2* which may be required to elicit its apoptotic response (Itoh *et al.*, 2004).

Very few of the genes identified in this study are differentially expressed in patients with 11q23 translocations. Apart from the *HOXA* and *PBX-3* genes, only *MT-1* and *ACTN4* are differentially expressed in patients with *MLL*-rearrangements (Armstrong *et al.*, 2002; Andersson *et al.*, 2005). Both *MT-1* and *ACTN4* are over-expressed in patients with 11q23 translocations. However, we found that these genes were up-regulated upon loss of MLL-ENL expression, indicating that their expression was suppressed by MLL-ENL. Several of the genes which were concordantly changed in both the constitutive and conditional cell lines upon the addition of dox are differentially expressed in patients. Both *SAP30* and *IRAK1BP* are up-regulated in patients with *MLL*-rearrangements (Andersson *et al.*, 2005). Accordingly, we found that these genes were down-regulated upon loss of MLL-ENL expression. Many of the published data sets obtained from patient samples include only the top 100 differentially expressed genes. Therefore, a more detailed analysis of the patient data may reveal a higher correlation between the genes differentially expressed in this study and in patients. However, it is possible that

some targets of the MLL-fusion proteins will not be identified from the analysis of patient material since leukaemic cells often contain multiple genetic aberrations which can result in varied and complex gene expression profiles.

Comparison of the genes identified in this study with those identified in a previous study of conditional MLL-ENL expression in primary haematopoietic progenitor cells (Zeisig *et al.*, 2004) revealed that *Hoxa9*, *Pbx-3* and *Cnn2* were the only differentially expressed genes common to both studies. The *Flt-3*, *Ptprc*, *Lmo2*, *Adam10*, *Meis-1* and *S100a4* genes have been previously identified as targets of MLL-ENL (Zeisig *et al.*, 2004) and are over-expressed in patients with 11q23 translocations (Armstrong *et al.*, 2002; Yeoh *et al.*, 2002; Andersson *et al.*, 2005). However, these genes were not identified as targets of MLL-ENL in this study. *Flt-3* and *Meis-1* were not expressed by the conditional cell lines. *Meis-1* was expressed by the constitutive cell line but this cell line did not express *Flt-3*. This result was surprising since it has been reported that Meis-1 activates the expression of *Flt-3* in Hoxa9 transformed cells (Wang *et al.*, 2005). It is possible that the level of Meis-1 expressed by the constitutive cell line is not sufficient to activate the expression of *Flt-3*. Although the expression of *Ptprc* and *Lmo2* decreased in all three conditional MLL-ENL cell lines upon loss of MLL-ENL expression, the fold changes did not reach statistical significance (data not shown). *S100a4* was not expressed by any of the lines and *Adam10* was not concordantly changed in each conditional cell line. For example, the expression of *Adam10* was unchanged in TRE-ME2, decreased in TRE-ME3 and increased in TRE-ME6 upon loss of MLL-ENL expression (data not shown). Hence, it is unlikely that *Adam10* is a target of MLL-ENL.

In the previous study of conditional MLL-ENL expression in primary HPCs, MLL-ENL was fused to the hormone binding domain of the oestrogen receptor such that MLL-ENL was activated upon the addition of tamoxifen (Zeisig *et al*, 2004). Immortalised myeloid cell lines were generated which were dependent on tamoxifen for their continued proliferation and survival. A potential caveat of this study is that differentially expressed genes were identified based on the analysis of only one cell line following treatment with or without tamoxifen and the effect that tamoxifen itself has on gene expression was not taken into account. Our experiment

was more stringent since three different cell lines were used to identify differentially expressed genes. Although the first 27aa of MLL was missing in the MLL-ENL cDNA that we used, our construct contained the entire ENL sequence that is found in MLL-ENL translocations in patients. In contrast, the previous study employed a truncated MLL-ENL cDNA encoding only the last 129 amino acids of ENL (aa 430-559 of ENL), which encompasses a conserved transactivation domain. Deletion mutagenesis studies revealed that this C-terminal domain of ENL was sufficient to transform haematopoietic progenitor cells when fused to MLL but the leukaemogenic potential of the truncated MLL-ENL fusion protein was not assessed (Slany *et al.*, 1998). A more recent study demonstrated that the N-terminus of ENL (amino acids 1 - 120) encodes a conserved YEATS domain which also confers transactivation properties to ENL and is required for histone binding (Zeisig *et al.*, 2005). Only the first five amino acids of ENL are missing in the full-length MLL-ENL fusion protein hence the YEATS domain is retained in the translocation. However, the YEATS domain is not retained in the truncated MLL-ENL fusion protein utilised by Zeisig *et al.* Since YEATS domain proteins are involved in histone modification in yeast (Bittner *et al.*, 2004; Zhang *et al.*, 2004), it is possible that the YEATS domain of ENL plays a role in chromatin remodelling and potentially in regulating target gene expression.

The Affymetrix analysis allowed us to identify genes regulated by MLL-ENL. These targets will allow us to delineate the molecular pathways de-regulated by expression of MLL-ENL and ascertain how MLL-ENL contributes to leukaemogenesis. Future work includes analysis of the role that the candidate MLL-ENL target genes play in leukaemogenesis. For the genes which were down-regulated following loss of MLL-ENL expression such as *Hoxa9* and *Pim-2*, this can be achieved by knocking down their expression in MLL-ENL immortalised cell lines by small-interfering RNA (siRNA). In contrast, investigation into the role of genes which were up-regulated following loss of MLL-ENL expression such as *Atrx* and *Ddb2*, will probably involve over-expressing these genes in the MLL-ENL immortalised cell lines. Ultimately, these target genes may provide exciting possibilities for the design of new leukaemia therapies.

Chapter 7 Establishment of a conditional model of MLL-ENL leukaemia *in vivo*.

Previous studies have shown that MLL-ENL immortalised cell lines can induce leukaemia with relatively short latency and high penetrance *in vivo* (Lavau *et al.*, 1997; Ayton and Cleary, 2003; Cozzio *et al.*, 2003). However, not all immortalised cell lines are leukaemogenic. For example, progenitors immortalised by Hoxa9 give rise to AML infrequently and only after an extremely long latency. The latency of Hoxa9 induced AML is dramatically shortened by the co-expression of Meis-1 (Kroon *et al.*, 1998; Wang *et al.*, 2005). Interestingly, progenitors immortalised by Hoxa9 and Meis-1 do not induce leukaemia *in vivo* if the immortalised cells were derived from progenitors cultured in GM-CSF (Calvo *et al.*, 2001). Since Hoxa9 and Meis-1 are thought to be key regulators of MLL-ENL mediated immortalisation (Zeisig *et al.*, 2004), it was important to determine whether the conditional MLL-ENL cell lines that we generated which lack *Meis-1* expression are leukaemogenic. If these cells could induce leukaemia *in vivo*, this would provide us with the opportunity to analyse whether continued MLL-ENL expression is required to maintain leukaemogenesis. Although it is known that MLL-ENL is required to maintain the phenotype of the immortalised cells *in vitro*, it is not known if continued expression is required to maintain the leukaemia *in vivo*. This is an important point to address since the immortalised cells generated *in vitro* may acquire secondary mutations following transfer into mice. These secondary mutations may be able to substitute for MLL-ENL expression. If this is the case the leukaemic cells may no longer be dependent on continued expression of the fusion protein and therapies designed to target MLL-ENL expression in humans may not be efficacious.

7.1 Transplantation of MLL-ENL immortalised cell lines into NOD/SCID mice induces AML.

All of the MLL-ENL immortalised cell lines were transferred into NOD/SCID mice in order to assess their leukaemogenic potential. The NOD/SCID strain was used since these mice are immunodeficient and are therefore unlikely to reject the donor immortalised cells which are from a C57Bl/6 background. All of the cell lines, with

the exception of TRE-ME6, induced leukaemia in recipient mice. Recipients of the constitutive cell line (c-ME1) developed AML with a shorter latency (80 ± 9 days, n = 5) than recipients of the conditional cell lines (Figure 7.1). Of the conditional cell lines, TRE-ME3 induced AML with a slightly shorter latency (118 ± 19 days, n = 5) than TRE-ME2 (132 ± 34 days, n = 9). None of the recipients of TRE-ME6 succumbed to AML within an observation period of over 300 days (Figure 7.1).

The leukaemic mice displayed minimal to extensive leukaemic infiltration of the liver and kidney (Figure 7.2). The leukaemic cells were also present in the lymph nodes and thymus in some cases. Analysis of the haematopoietic organs of leukaemic mice by flow cytometry revealed that all of the leukaemias were derived from donor cells. The donor and host cells could be distinguished since the haematopoietic cells of the C57Bl/6 mice express CD45.2, whereas the haematopoietic cells of the NOD/SCID mice express CD45.1. The surface marker expression profile and morphology of the bone marrow and spleen from a representative c-ME1 recipient is shown in Figure 7.3. Both the bone marrow and spleen were composed almost exclusively of donor cells which expressed Mac-1, Gr-1 and intermediate levels of c-Kit (Figure 7.3A). This expression profile was very similar to that of the original c-ME1 cell line prior to transfer (Figure 5.5B). Morphological analysis revealed that the majority of the leukaemic cells in the spleen were myeloblasts while the bone marrow contained a mixture of myeloblasts and more differentiated myeloid cells (Figure 7.3B). The fact that differentiated myeloid cells were present in the bone marrow and that these were donor derived proves that c-ME1 does possess some differentiation capacity *in vivo*. This data is consistent with the leukaemia being classified as a myeloid leukaemia with maturation according to the Bethesda mouse leukaemia classification guidelines (Kogan *et al.*, 2002).

The conditional MLL-ENL cells could be distinguished from host cells based on their expression of EGFP, since tTA expression is linked via an IRES to EGFP. An analysis of the spleen and bone marrow of a representative TRE-ME2 recipient is shown in Figure 7.4. In this mouse, half of the cells in the bone marrow expressed EGFP and were therefore donor derived, whereas the majority of splenocytes (75%) were donor derived (Figure 7.4). In contrast to the parental TRE-ME2 cell line

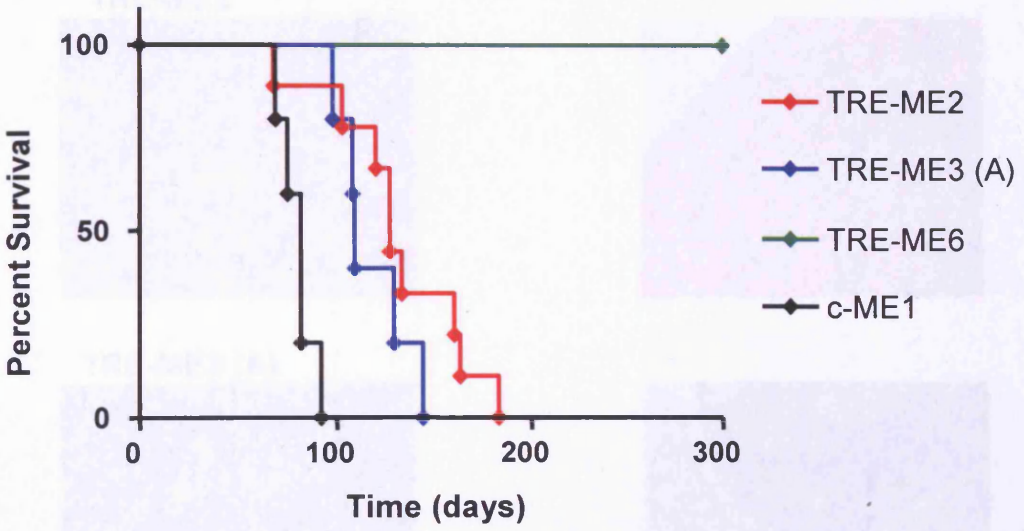

Figure 7.1 Leukaemogenicity of the MLL-ENL cell lines. 10^6 TRE-ME2 (red diamonds), TRE-ME3(A) (blue diamonds), TRE-ME6 (green diamonds) and c-ME1 (black diamonds) immortalised cells were injected intraperitoneally into NOD/SCID mice. The mice were sacrificed when they began to show signs of illness (section 2.11).

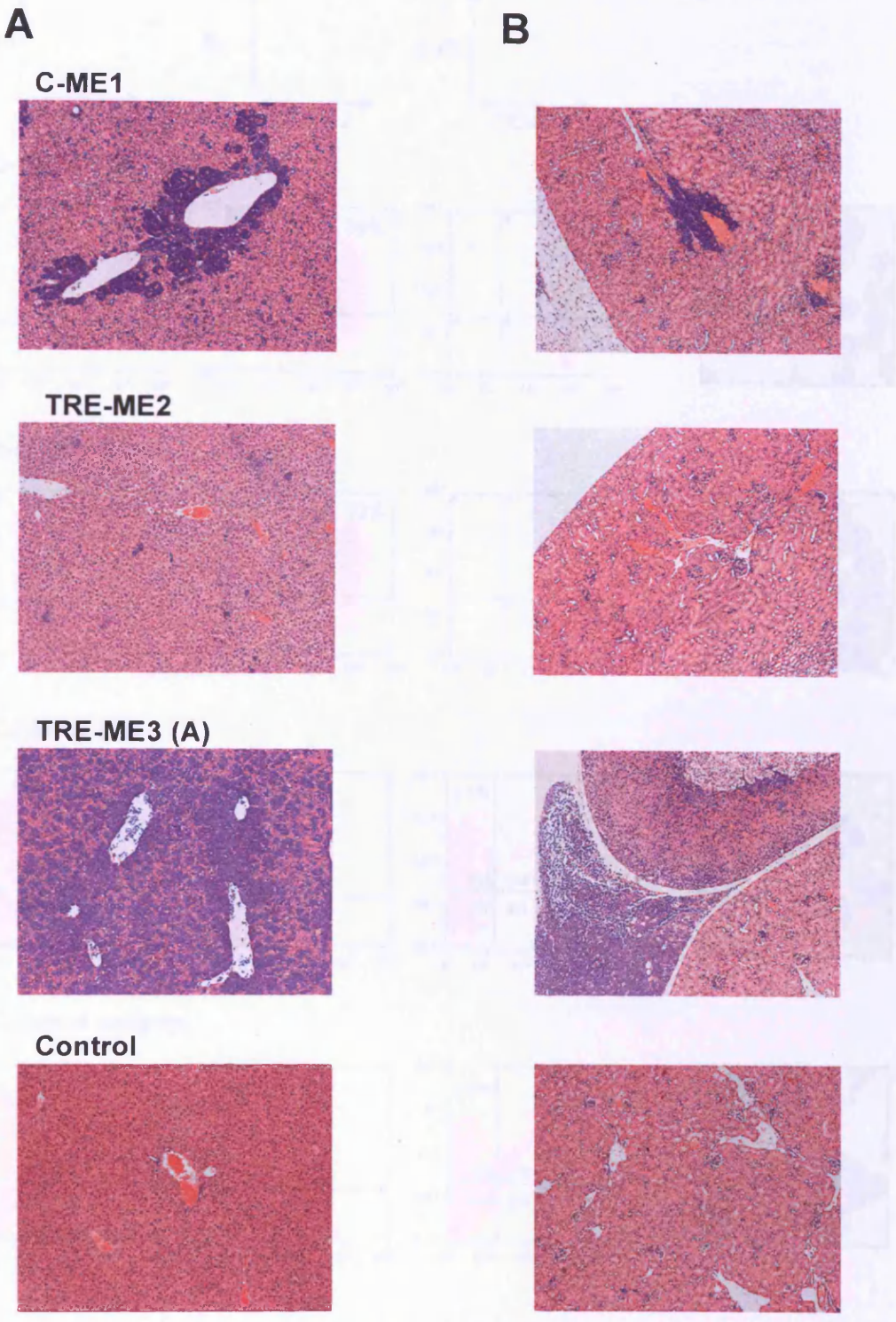

Figure 7.2 Leukaemic infiltration of the liver and kidney. 10^6 immortalised cells were injected into NOD/SCID mice which were sacrificed when they began to show signs of ill health. **A)** The liver and **B)** kidney of moribund mice were fixed in formalin, sectioned and stained with hematoxylin and eosin. A healthy aged matched mouse served as a negative control. Original magnification x 40.

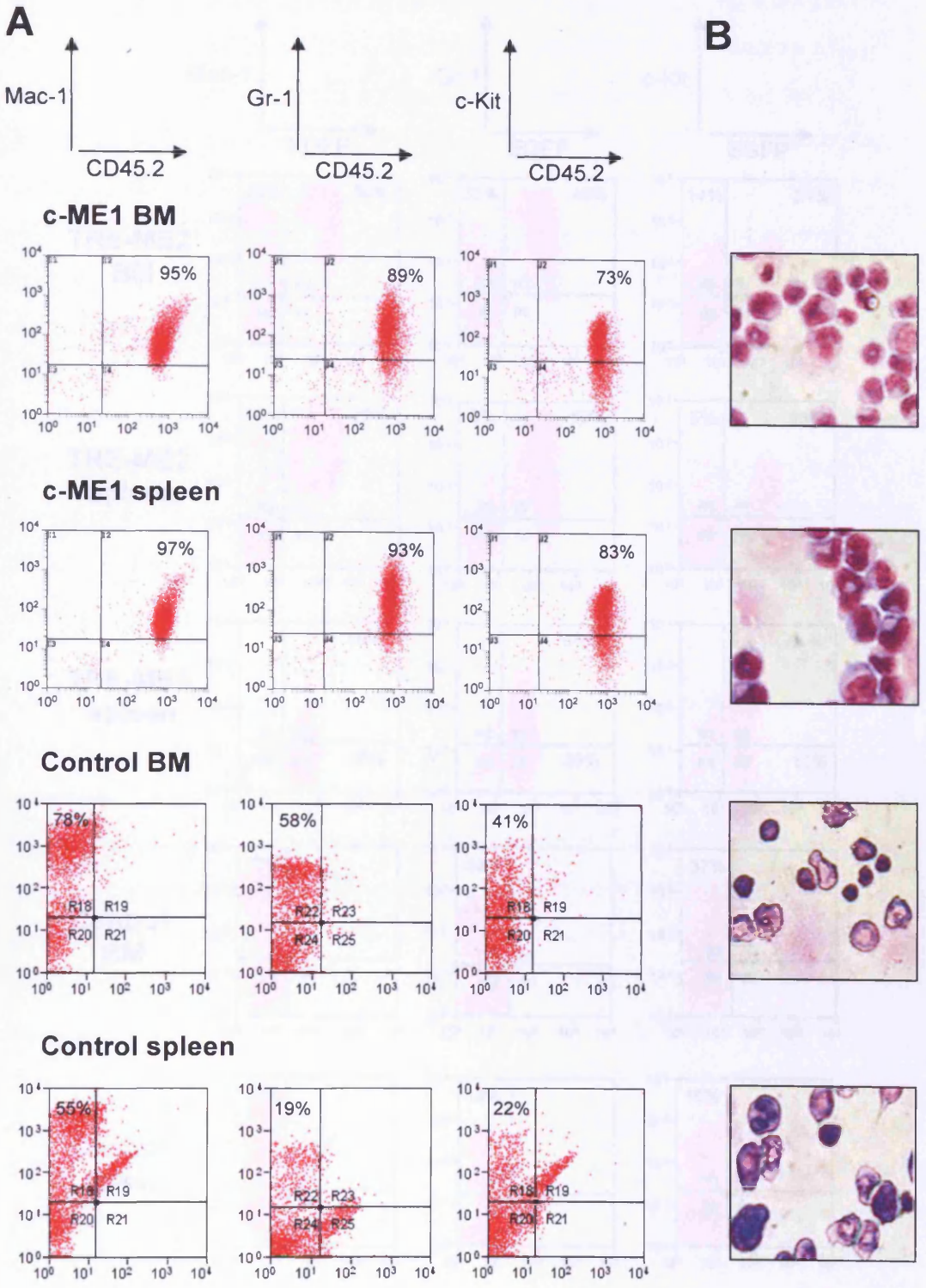

Figure 7.3 C-ME1 is leukaemogenic in NOD/SCID mice. The leukaemogenic potential of the constitutive cell line was examined by transfer into NOD/SCID mice. Moribund mice were sacrificed and the spleen and bone marrow were lysed and analysed by **A)** flow cytometry and **B)** cytospin analysis followed by MGG staining. The plots show the expression of the Mac-1, Gr-1 and c-Kit cell surface antigens versus the CD45.2 antigen. CD45.2 is only expressed by donor cells. A healthy age-matched mouse served as a negative control.

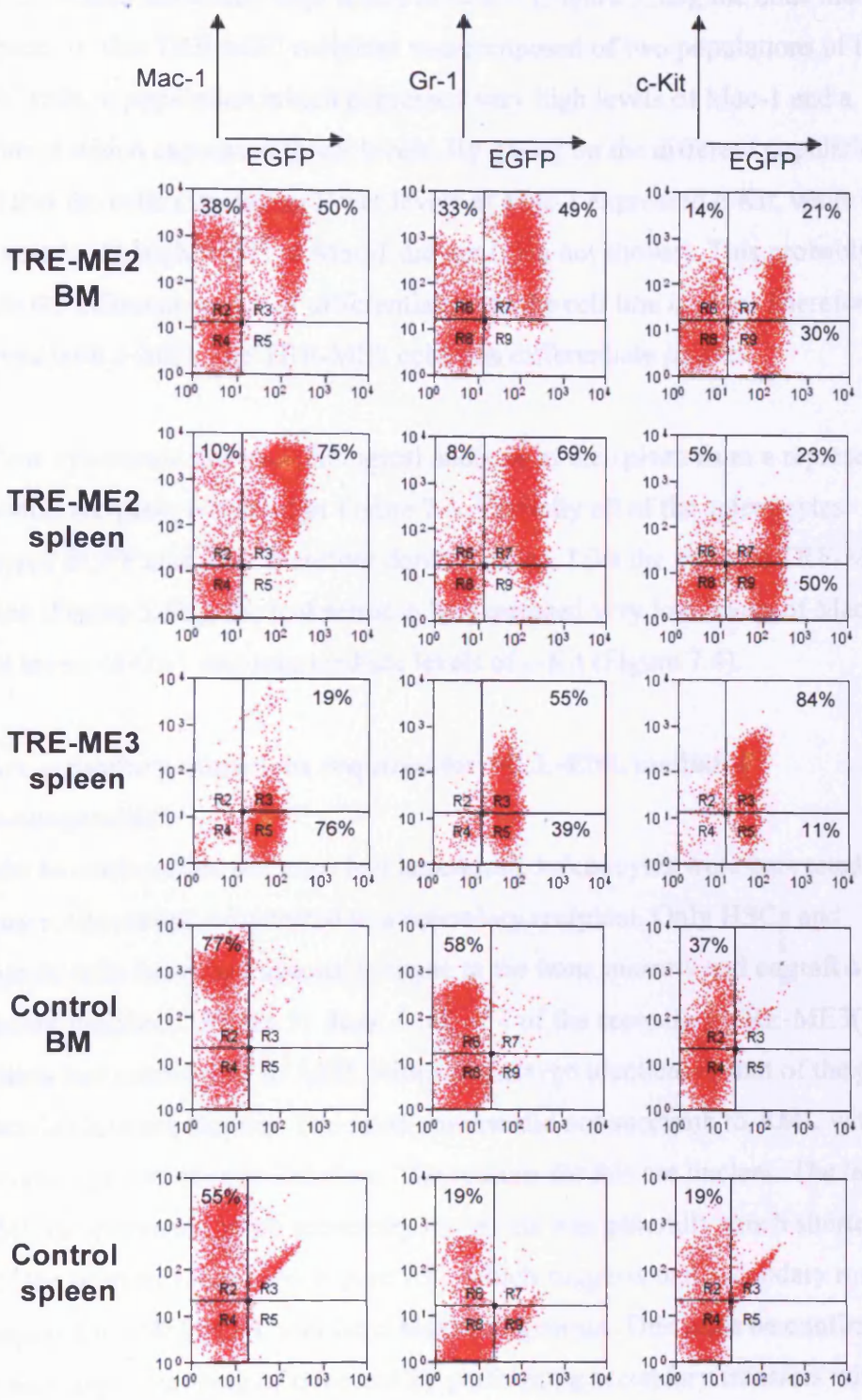

Figure 7.4 TRE-ME2 and TRE-ME3(A) are leukaemogenic in NOD/SCID mice. The leukaemogenic potential of TRE-ME2 and TRE-ME3(A) were examined by transfer into NOD/ SCID mice. Moribund mice were sacrificed and the spleen and bone marrow were analysed by flow cytometry. The plots show the expression of the Mac-1, Gr-1 and c-Kit cell surface antigens versus EGFP. EGFP is only expressed by donor cells. A healthy age-matched control mouse served as a negative control.

which expressed uniformly high levels of Mac-1 (Figure 5.5B), the bone marrow and spleen of this TRE-ME2 recipient was composed of two populations of $EGFP^+$ $Mac\text{-}1^+$ cells, a population which expressed very high levels of Mac-1 and a population which expressed lower levels. By gating on the different populations, we found that the cells expressing lower levels of Mac-1 expressed c-Kit, while the cells expressing high levels of Mac-1 did not (data not shown). This probably reflects the different stages of differentiation of the cell line *in vivo*. Therefore, as observed with c-ME1, the TRE-ME2 cells can differentiate *in vivo*.

The flow cytometric and morphological analysis of the spleen from a representative TRE-ME3 recipient is shown in Figure 7.4. Virtually all of the splenocytes expressed EGFP and were therefore donor derived. Like the parental TRE-ME3(A) cell line (Figure 5.5B), the leukaemic cells expressed very low levels of Mac-1, higher levels of Gr-1 and intermediate levels of c-Kit (Figure 7.4).

7.2 Are secondary mutations required for MLL-ENL mediated leukaemogenesis?

In order to confirm that the mice had leukaemia, splenocytes were harvested from a primary recipient and transferred to a secondary recipient. Only HSCs and leukaemic cells have the capacity to home to the bone marrow and engraft a secondary recipient. Within 51 days, 3 out of 4 of the secondary TRE-ME3(A) recipients had succumbed to AML with a phenotype identical to that of the primary leukaemia (data not shown). The fourth mouse did not succumb to AML within an observation period of over 150 days. The reasons for this are unclear. The latency of AML development in the secondary recipients was generally much shorter than that of the primary recipients (Figure 7.5), which suggests that secondary mutations are required for MLL-ENL mediated leukaemogenesis. This must be confirmed by analysing larger numbers of mice and by performing secondary transfers with splenocytes harvested from primary recipients of the other immortalised cell lines.

7.3 Is continued MLL-ENL expression required to maintain AML *in vivo*?

Having established that TRE-ME2 and TRE-ME3(A) are leukaemogenic, we wanted to examine whether leukaemic mice could be cured by administering dox. Since TRE-ME3(A) dies rather than terminally differentiating upon the addition of

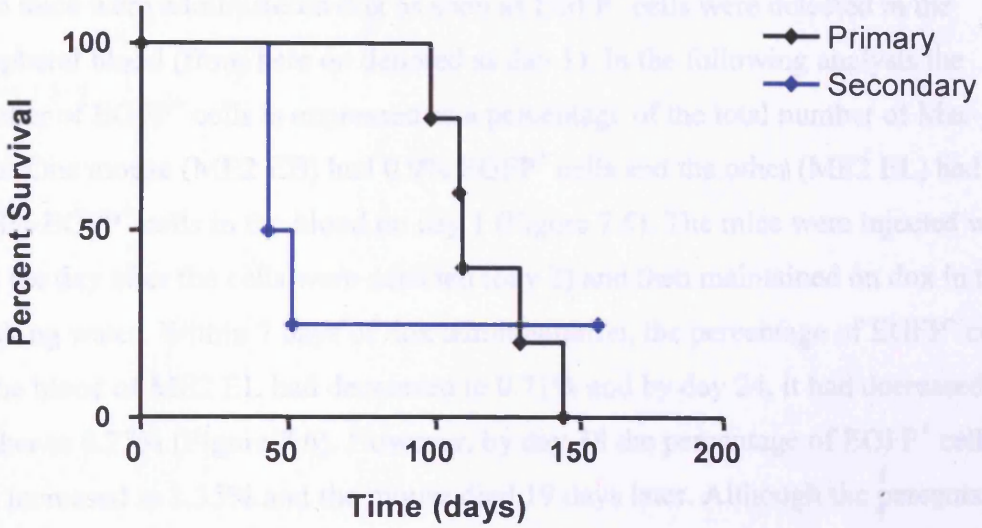

Figure 7.5 Development of leukaemia in primary and secondary recipients of TRE-ME3(A) immortalised cells. 10^6 TRE-ME3(A) immortalised cells were injected into NOD/SCID mice. Primary recipients (black diamonds) were sacrificed when they began to show signs of ill health. 2 x 10^6 or 10^7 splenocytes from a primary recipient were injected into secondary recipients (blue diamonds) which were sacrificed when they began to show signs of illness.

dox *in vitro*, we performed preliminary experiments with TRE-ME2. Nine mice were injected with the TRE-ME2 cell line. The mice were monitored for leukaemia development by analysing the EGFP expression of peripheral blood cells at regular intervals post transfer. The mean time-frame from injection to the detection of EGFP positive cells in the peripheral blood was 97 ± 10 days (n = 7). Two mice died of leukaemia without prior detection of cells in the blood. This may have been because the immortalised cells were able to grow as solid masses in the intraperitoneal cavity of some of the mice. Therefore, it is likely that the mice died as a result of the solid tumour, before the leukaemia was fully established.

Two mice were administered dox as soon as $EGFP^+$ cells were detected in the peripheral blood (from here on denoted as day 1). In the following analysis the number of $EGFP^+$ cells is expressed as a percentage of the total number of $Mac\text{-}1^+$ cells. One mouse (ME2 EB) had 0.9% $EGFP^+$ cells and the other (ME2 EL) had 1.28% $EGFP^+$ cells in the blood on day 1 (Figure 7.6). The mice were injected with dox the day after the cells were detected (day 2) and then maintained on dox in the drinking water. Within 7 days of dox administration, the percentage of $EGFP^+$ cells in the blood of ME2 EL had decreased to 0.71% and by day 24, it had decreased further to 0.27% (Figure 7.6). However, by day 38 the percentage of $EGFP^+$ cells had increased to 1.35% and the mouse died 19 days later. Although the percentage of $EGFP^+$ cells in the peripheral blood of ME2 EB increased initially with dox treatment, the percentage decreased with time and by day 24, the percentage of $EGFP^+$ cells in the peripheral blood was half of that on day 7. However, by day 38 the number of $EGFP^+$ cells in the peripheral blood had increased significantly to 66% and the mouse died 8 days later (Figure 7.6). Although dox did not cure the mice, it substantially hindered the progression of the leukaemia since mice which received dox survived for a lot longer (51 ± 8 days, n = 2) after the detection of $EGFP^+$ cells in the peripheral blood than mice which did not receive dox (24 ± 4 days, n = 3) (Figure 7.7).

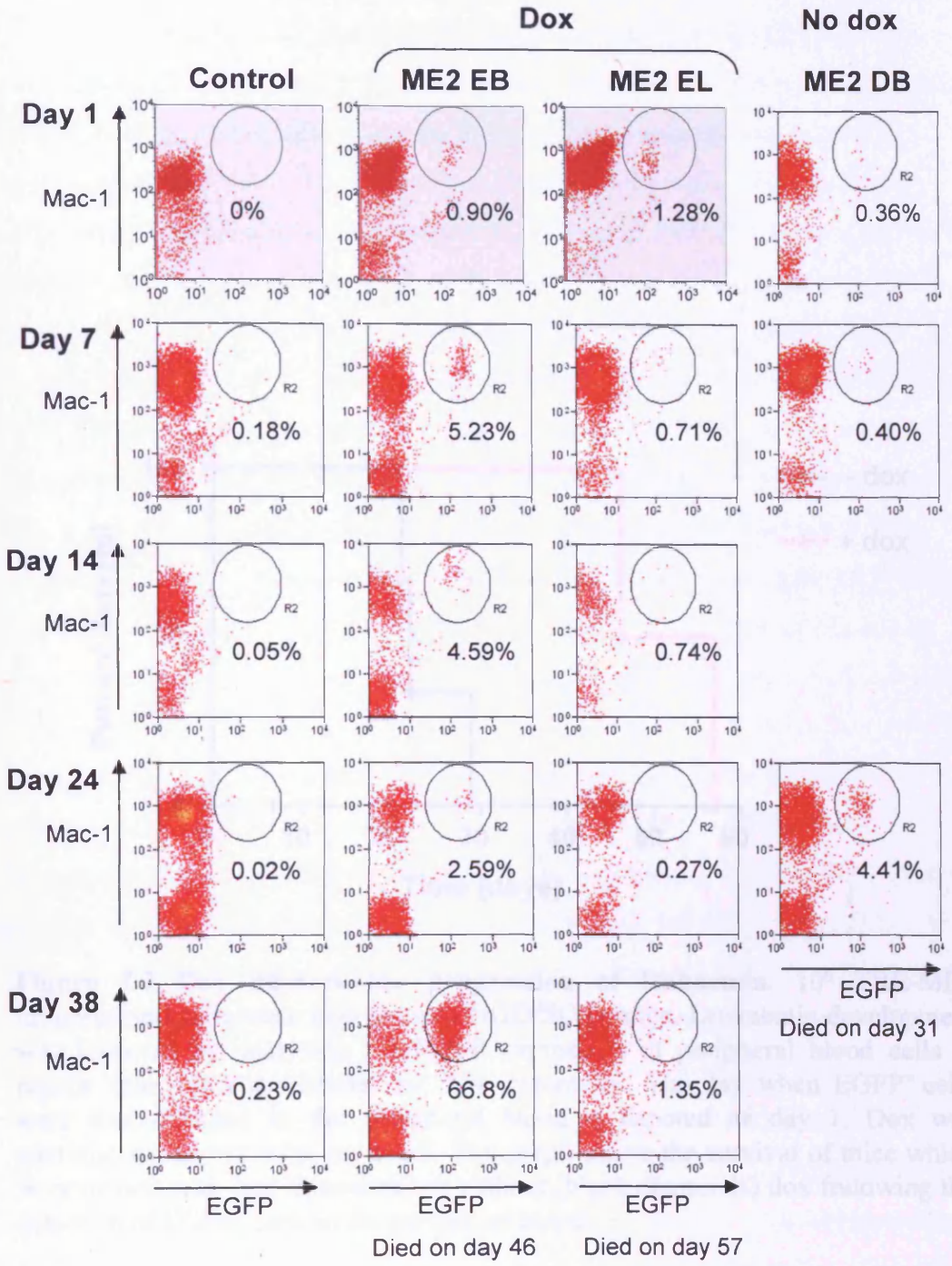

Figure 7.6 Reversing the leukaemia with dox. 10^6 TRE-ME2 immortalised cells were injected into NOD /SCID mice. Leukaemia development was followed by analysing the Mac-1 and EGFP expression of peripheral blood cells at regular intervals by flow cytometry. The day when EGFP positive cells were first detected in the peripheral blood of each mouse is denoted as day 1. Two mice (ME2 EB and ME2 EL) were administered dox on day 2 whereas ME2 DB was not given dox. Leukaemia progression was followed by analysing the percentage of Mac-1 expressing cells that were EGFP positive at regular intervals by flow cytometry. A control mouse which did not receive TRE-ME2 cells served as a negative control.

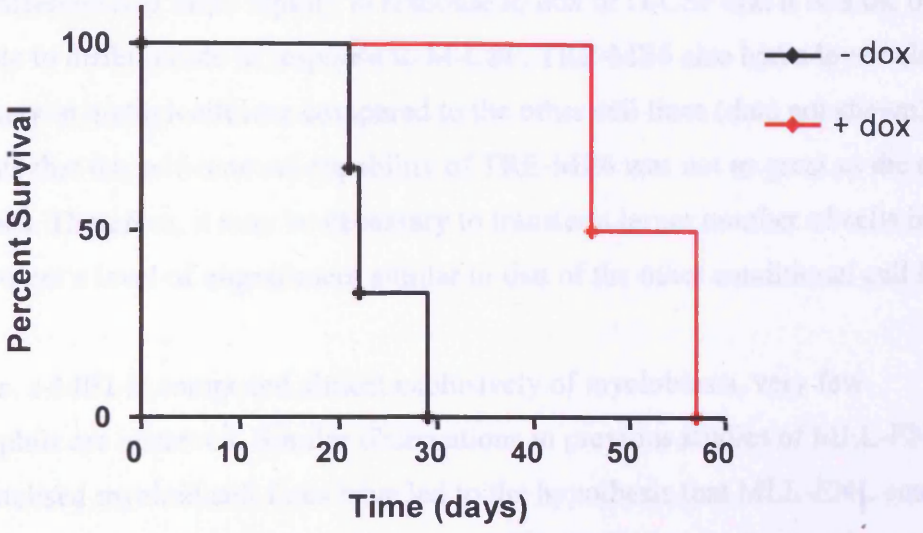

Figure 7.7 Dox hinders the progression of leukaemia. 10^6 TRE-ME2 immortalised cells were injected into NOD/SCID mice. Leukaemia development was followed by analysing the EGFP expression of peripheral blood cells at regular intervals post transfer by flow cytometry. The day when $EGFP^+$ cells were first detected in the peripheral blood is denoted as day 1. Dox was administered to two mice on day 2. The graph shows the survival of mice which were treated with (red diamonds) or without (black diamonds) dox following the detection of $EGFP^+$ cells in the peripheral blood.

Discussion

All of the MLL-ENL immortalised cell lines, with the exception of TRE-ME6, were leukaemogenic in NOD/SCID mice. Recipients of the constitutive cell line (c-ME1) developed AML with a latency similar to that previously reported for recipients of MLL-ENL immortalised cell lines (Lavau *et al.*, 1997), whereas recipients of the conditional cell lines TRE-ME2 and TRE-ME3, developed AML with a slightly longer latency. TRE-ME6 did not induce AML over the observed time frame of 7 months. This may be because the differentiation impairment imposed by MLL-ENL is less severe in TRE-ME6 than the other cell lines. TRE-ME6 differentiated more rapidly in response to dox or G-CSF and it was the only line able to differentiate in response to M-CSF. TRE-ME6 also had a lower cloning efficiency in methylcellulose compared to the other cell lines (data not shown). This suggests that the self-renewal capability of TRE-ME6 was not as great as the other cell lines. Therefore, it may be necessary to transfer a larger number of cells in order to get a level of engraftment similar to that of the other conditional cell lines.

In vitro, c-ME1 is composed almost exclusively of myeloblasts, very few neutrophils are observed. Similar observations in previous studies of MLL-ENL immortalised myeloid cell lines have led to the hypothesis that MLL-ENL causes a block in myeloid differentiation. However, MLL-ENL immortalised cells can be induced to differentiate in response to G-CSF. Furthermore, the leukaemic cells are able to differentiate *in vivo*, since the bone marrow of a leukaemic mouse, which was composed almost exclusively of donor c-ME1 cells, consisted of a heterogeneous population of myeloblasts, myelocytes and neutrophils. Therefore, rather than causing an absolute differentiation block, MLL-ENL may suppress the differentiation of myeloid progenitors, which when combined with an enhanced proliferation, survival and self-renewal capacity ultimately results in leukaemogenesis.

The TRE-ME2 and TRE-ME3 conditional cell lines express *Meis-2* rather than *Meis-1*, yet they are still capable of inducing leukaemia *in vivo*. This suggests that Meis-1 is not required for MLL-ENL induced leukaemogenesis. Although it has not been shown whether Meis-2 can accelerate Hoxa9 induced leukaemogenesis, it is possible that *Meis-2* is able to compensate for the absence of *Meis-1* expression in

these cells. However, the latency of leukaemia induced by these cell lines is slightly longer than that of the constitutive cell line which expresses *Meis-1*. Therefore, although Meis-2 may be able to compensate for Meis-1, it may not be as efficient as Meis-1 at accelerating Hox induced leukaemogenesis.

Preliminary experiments revealed that secondary TRE-ME3(A) recipients developed AML with a shorter latency than that of primary recipients. This suggests that other genetic events occur which co-operate with MLL-ENL to induce AML. This hypothesis is supported by the multi-step model of MLL-AF9 leukaemogenesis, which is based on the finding that MLL-AF9 knock-in mice develop a myeloproliferative disease prior to the onset of AML (Dobson *et al.*, 1999). The myeloproliferative disease commences during the prenatal phase which is followed by an increased self-renewal or survival capacity of myeloid progenitors in the post-natal phase and eventual AML development in the adult phase (Johnson *et al.*, 2003). The long latency of AML development and the fact that it is preceded by a myeloproliferative disease suggests that secondary mutations are required for MLL-AF9 mediated leukaemogenesis.

Flt-3 gene mutations are possible candidates for co-operating secondary genetic events in *MLL*-rearranged leukaemias. In paediatric lymphoid leukaemias, a correlation exists between *MLL*-translocations and point mutations in the *Flt3* gene (Taketani *et al.*, 2004). Although internal tandem duplications of the juxtamembrane domain of the *Flt3* gene (*Flt3*-ITDs) are frequently found in AML patients (Stirewalt and Radich, 2003), an association between *Flt3*-ITDs and duplications or double strand breaks in the *MLL* gene has been reported (Libura *et al.*, 2003). Furthermore, a recent study demonstrated that Flt3-ITD co-operated with MLL-ENL to induce leukaemogenesis in mice, since co-expression of *MLL-ENL* and *Flt3*-ITD dramatically shortened the latency of MLL-ENL induced AML from 92 days to just 17 days (Ono *et al.*, 2005).

Although most cancers are the result of multiple genetic lesions, the inactivation of only one genetic lesion can induce tumour regression (Felsher and Bishop, 1999; Huettner *et al.*, 2000). MYC transgenic mice, which were generated using the tetracycline regulatory system, died of T cell lymphomas or AML in the absence of

dox (Felsher and Bishop, 1999). Treatment of moribund mice with dox resulted in sustained tumour regression as a result of tumour cell differentiation. Similarly, BCR-ABL transgenic mice were effectively cured of ALL as a result of BCR-ABL inactivation by tetracycline (Huettner *et al.*, 2000). Therefore, in these models, Myc and BCR-ABL are required to initiate as well as maintain leukaemogenesis. We examined whether continued expression of MLL-ENL was required to maintain leukaemogenesis by using dox to turn off MLL-ENL expression in mice that showed early signs of leukaemia. Initially dox caused a decrease in the number of leukaemic cells in the blood. However, the effect was not sustained and the mice eventually died of AML, although with a longer latency than mice which did not receive dox. Therefore, dox has a transient effect on decreasing the leukaemic burden.

In order to confirm that dox hinders leukaemia progression, the experiment must be repeated using larger numbers of mice. Transfer of the cells by intravenous rather than intraperitoneal injection may better model the disease since the mice would no longer develop tumours in the intraperitoneal cavity. Importantly, dox should be administered to recipients of the constitutive cell line in order to assess whether the delay in leukaemia progression is specific to the conditional mice. Since dox decreases the proliferation rate of the constitutive immortalised cell line *in vitro*, it is possible that the observed delay in disease progression is due to the effect of dox on slowing the proliferation of the leukaemic cells. If this is the case we would expect to observe a similar delay in disease progression in recipients of the constitutive cell line. In order to assess whether the leukaemic cells from the conditional mice retain the ability to differentiate upon loss of MLL-ENL expression, a cell line will be established from the leukaemic cells and the ability of dox to induce differentiation will be examined *in vitro*.

Chapter 8 Conclusions

In order to generate a conditional system of MLL-ENL expression in primary HPCs, the retroviral delivery of the Tet-Off system to target cells was optimised using two reporter constructs. Conditional reporter gene expression was achieved in both NIH3T3 cells and HPCs, although a low level of tTA independent reporter gene expression was observed. Subsequent experiments revealed that tTS was able to prevent tTA independent reporter gene expression and this was regulatable by dox. We concluded that MLL-ENL expression may be regulated by tTS if low levels of tTA independent MLL-ENL expression are sufficient to immortalise HPCs. Conditional MLL-ENL expression was achieved following co-transduction of NIH3T3 cells with the conditional MLL-ENL and tTA expression constructs. Therefore, experiments were undertaken to generate constitutive and conditional MLL-ENL immortalised cell lines derived from HPCs isolated from E12 foetal liver.

The constitutive MLL-ENL expression construct immortalised c-Kit$^+$ Ter-119$^-$ and Sca-1$^+$ c-Kit$^+$ foetal HPCs under myeloid conditions and immortalised myeloid cell lines were established in liquid culture. In contrast, the constitutive MLL-AF4 expression construct did not immortalise c-Kit$^+$ Ter-119$^-$ foetal HPCs under myeloid or lymphoid conditions. Although three immortalised cell lines were generated from foetal HPCs transduced with the constitutive MLL-ENL expression construct, the conditional MLL-ENL and tTA expression constructs did not immortalise these progenitors. However, the conditional MLL-ENL and tTA expression constructs were able to immortalise unsorted or lin$^-$ HPCs isolated from the bone marrow of 5-FU treated mice.

HPCs immortalised using the conditional MLL-ENL and tTA expression constructs failed to self-renew in methylcellulose upon loss of MLL-ENL expression. Furthermore, conditional immortalised myeloid cell lines either terminally differentiated into neutrophils and macrophages or died upon loss of MLL-ENL expression. Thus we concluded that continued MLL-ENL expression is required to maintain the immortalised phenotype *in vitro*. Three conditional immortalised cell lines and one constitutive cell line, which was derived from c-Kit$^+$ bone marrow

isolated from 5-FU treated mice, were characterised in detail. All of the cell lines possessed slightly different immunophenotypes suggesting that the pool of progenitors susceptible to transformation by MLL-ENL is heterogeneous. Clones of one of the conditional cell lines possessed neutrophil and macrophage differentiation potential in response to G-SCF and M-CSF, respectively. Hence, the differentiation block imposed by MLL-ENL can occur prior to commitment to the granulocyte and monocyte lineages.

Global *Hox* gene expression profiling revealed that MLL-ENL maintained the aberrant expression of multiple *Hoxa* genes and the Hox co-factor *Pbx-3*. In contrast to previous studies, we found that Meis-1 was not a critical mediator of MLL-ENL activity since *Meis-1* was not expressed by any of the conditional cell lines. *Meis-2* was expressed instead of *Meis-1* in these cells, yet *Meis-2* was not regulated by MLL-ENL. Hence, it is possible that MLL-ENL activity is mediated by multiple Hoxa proteins which synergise with Pbx-3 and the Meis Hox co-factors normally expressed in myeloid progenitors. Consequently, rather than targeting individual *Hox* genes, therapies which target the pathways regulated by multiple *Hox* genes, acting in concert, may be more efficacious.

The constitutive cell line and two out of the three conditional cell lines induced leukaemia *in vivo*. Preliminary experiments revealed that loss of MLL-ENL expression *in vivo* resulted in a transient decrease in the leukaemic burden. However, the effect was not sustained and the mice eventually died of AML. Secondary transfer experiments suggest that in addition to MLL-ENL, other mutations may be required to induce leukaemia *in vivo*. Further experiments are required in order to determine whether these secondary mutations are able to substitute for MLL-ENL expression and therefore whether therapies that target MLL-ENL expression will be efficacious.

Although the de-regulation of *Hoxa* gene expression may be central to MLL-ENL leukaemogenesis, these genes are de-regulated in the leukaemic cells of many AML patients which lack *MLL*-rearrangements. Therefore, MLL-ENL must confer other properties to the leukaemic cells which underlie the poor prognosis of leukaemias bearing this fusion protein. Global gene expression profiling revealed that MLL-

ENL may confer a protection from apoptosis by activating the expression of *Pim-2* and repressing the expression of multiple mediators of apoptosis such as *Ddb2*, *Aatk* and *Bri3bp*. Furthermore, MLL-ENL may maintain or activate the expression of *Aldh1* which confers resistance to cytotoxic drugs. The aberrant expression of these genes by the leukaemic cells may contribute to the poor prognosis of t(11;19) leukaemias. Ultimately, these MLL-ENL target genes may offer new possibilities for leukaemia therapy.

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Appendix

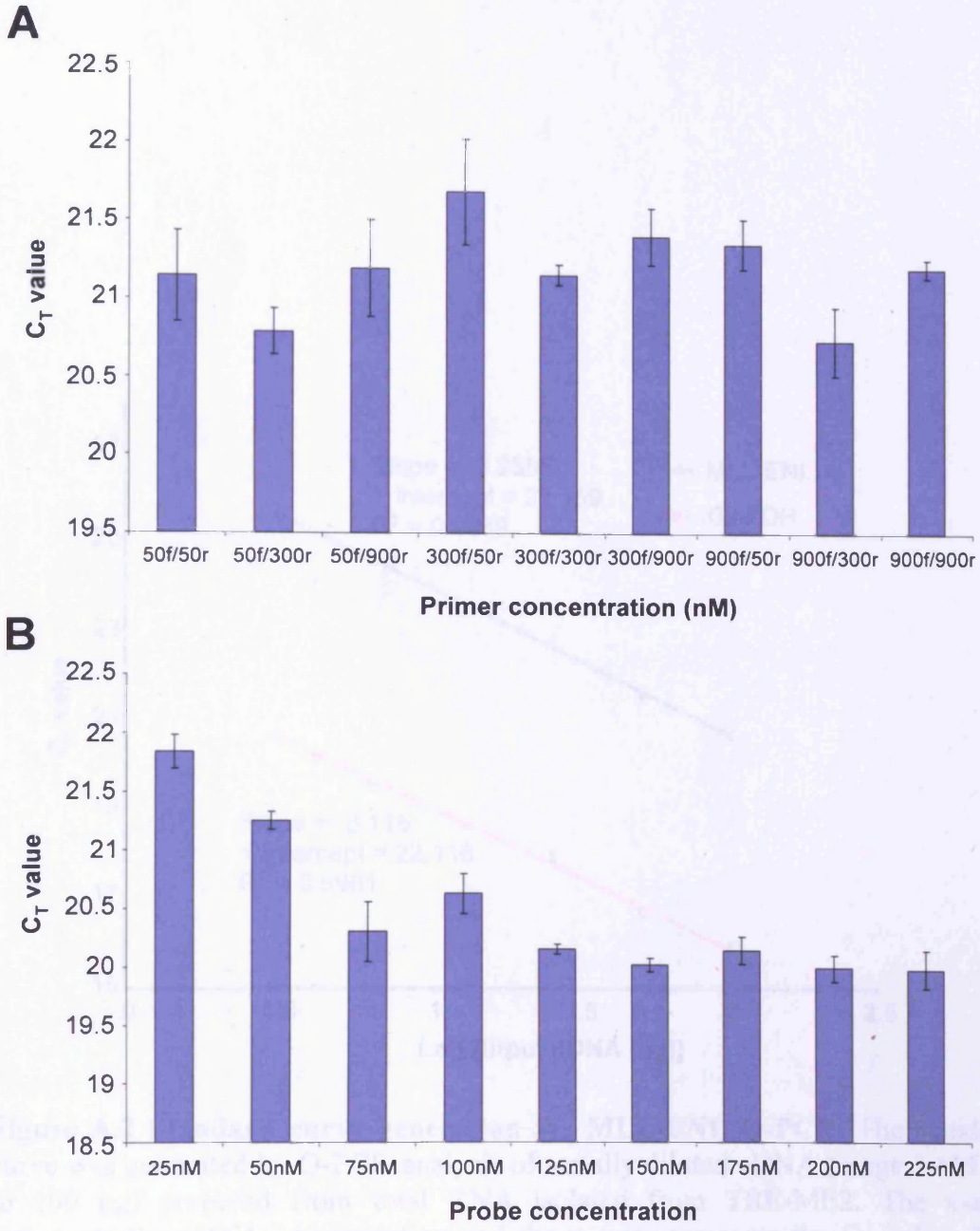

Figure A.1 MLL-ENL Q-PCR probe and primer optimisation. A probe and primer set was designed which spanned the MLL-ENL breakpoint in order to measure MLL-ENL transcript expression by Q-PCR. **A)** The optimal primer concentration and **B)** the optimal probe concentration were determined by amplifying the MLL-ENL break-point sequence from cDNA using various concentrations of forward primer (f), reverse primer (r) or probe. The optimum primer and probe concentrations were determined by plotting the cycle threshold (C_T) value versus primer / probe concentration. The optimal primer combination was 900f/300r (900 nM forward primer and 300 nM reverse primer). This combination produced the lowest C_T value and the highest reaction (Rn) value. The optimal probe concentration was 150 nM since this was the lowest concentration which did not affect the C_T value.

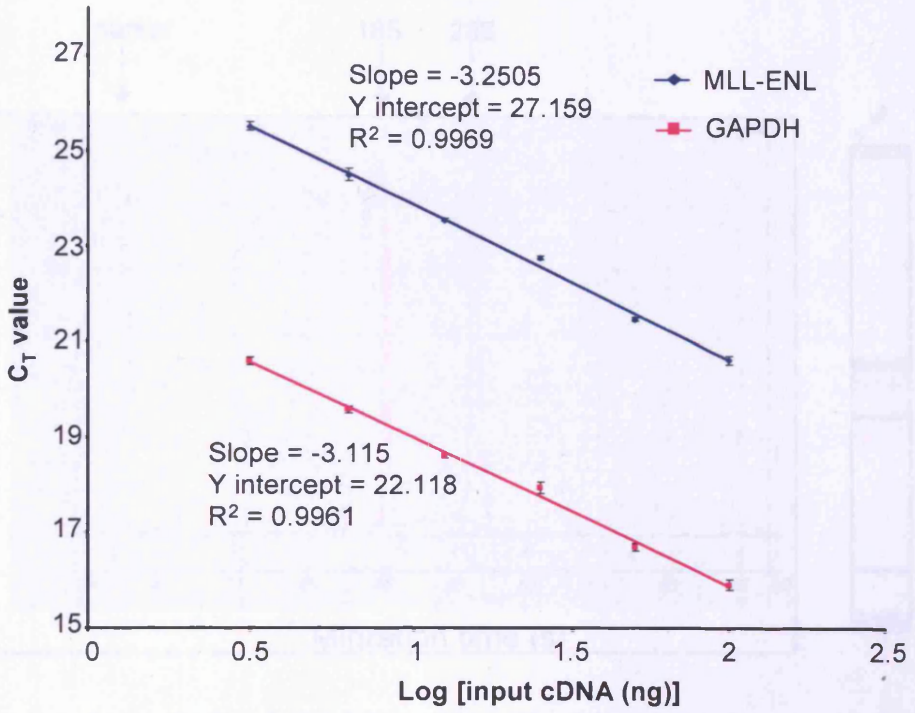

Figure A.2 Standard curve generation for MLL-ENL Q-PCR. The standard curve was generated by Q-PCR analysis of serially diluted cDNA (range 3.125 ng to 100 ng) prepared from total RNA isolated from TRE-ME2. The x-axis represents the cDNA concentration and the y-axis represents the C_T value. The slope reflects the kinetics of the assay, the y intercept reflects the sensitivity of the assay and the correlation co-efficient R^2 indicates the reproducibility of the assay at high and low cDNA concentrations.

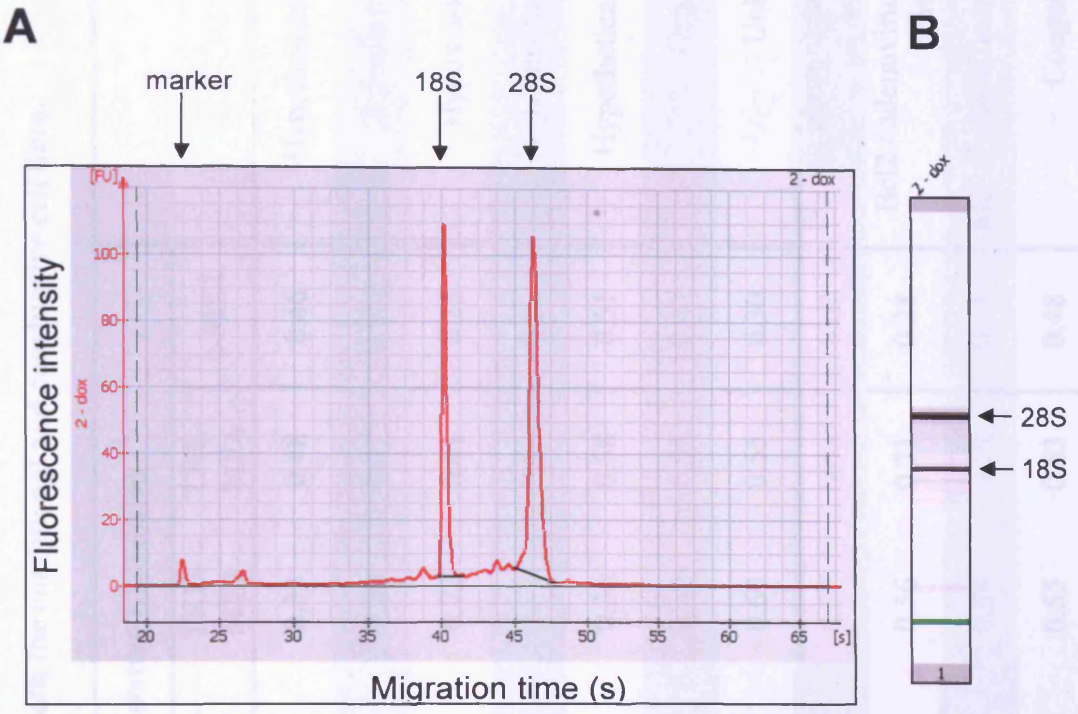

Figure A.3 Analysis of RNA integrity. The RNA integrity was analysed using the Agilent 2100 Bioanalyser according to the manufacturer's instructions. **A)** A representative electropherogram showing the fluorescence intensity versus migration time. The ratio of the area under the 28S and 18S ribosomal peaks is an indication of the integrity of the RNA. A high quality RNA sample typically has a ratio of 28S:18S ribosomal peaks of 2:1. A ratio between 1.5:1 and 2:1 is acceptable for Affymetrix analysis. The sample shown has a ratio of 1.6:1. **B)** A representative densitometry plot for the same sample shown in **A**.

Table A.1 Genes down-regulated by dox in both the conditional and constitutive cell lines.

Gene	P-value	Relative Expression					Product
		Average RE	TRE-ME2	TRE-ME3	TRE-ME6	c-ME1	
3110057O12Rik	0.0364	0.35	0.25	0.33	0.48	0.46	Hypothetical protein LOC269423
Selenbp1	0.0469	0.37	0.13	0.50	0.47	0.083	Selenium binding protein 1
2310016C08Rik	0.0218	0.46	0.26	0.72	0.41	0.35	Hypoxia-inducible protein 2
Egln1	0.0196	0.48	0.30	0.55	0.60	0.32	EGL nine homolog 1 (C.elegans)
C030034I22Rik	0.0339	0.48	0.50	0.36	0.58	0.51	Hypothetical protein LOC77533
Gm446	0.0497	0.50	0.39	0.42	0.69	0.41	Gene model 446
A130078K24Rik	0.0287	0.50	0.35	0.60	0.55	0.30	Unknown EST
Pppr13b	0.00567	0.50	0.45	0.37	0.68	0.62	Protein phosphatase 1, regulatory (inhibitor) subunit 3B
Bnip3	0.0474	0.51	0.26	0.56	0.71	0.38	Bcl2 / adenovirus E1b 19kDa-interacting protein 1, NIP3
Mns1	0.028	0.51	0.56	0.38	0.59	0.41	Meiosis-specific nuclear structural protein 1
F10	0.0471	0.53	0.41	0.55	0.63	0.48	Coagulation factor X

AI314180	0.00966	0.54	0.47	0.57	0.58	0.34	Expressed sequence AI314180
Ang1	0.0412	0.56	0.35	0.45	0.89	0.07	Angiogenin, ribonuclease A family, member 1
Ptdsr	0.00611	0.58	0.67	0.53	0.55	0.21	Phosphatidylserine receptor
Tiparp	0.0444	0.59	0.48	0.48	0.82	0.54	TCDD-inducible poly (ADP- ribose) polymerase
Irak1bp	0.0196	0.59	0.65	0.59	0.54	0.71	Interleukin-1 receptor-associated kinase 1 binding protein 1
Gpatc4	0.0148	0.59	0.57	0.68	0.51	0.40	G patch domain containing 4
Sap30	0.0474	0.60	0.47	0.53	0.81	0.52	Sin3 associated polypeptide
Zfp259	0.00583	0.60	0.53	0.64	0.64	0.35	Zinc finger protein 259
Fndc3a	0.0385	0.60	0.50	0.67	0.64	0.34	Fibronectin type III, domain containing 3a
1700022C21Rik	0.0303	0.61	0.53	0.55	0.74	0.11	Hypothetical protein LOC76416
2410025L10Rik	0.0325	0.61	0.68	0.53	0.63	0.34	Hypothetical protein LOC381668
Frap1	0.0208	0.62	0.85	0.54	0.48	0.64	FK506 binding protein 12-rapamycin associated protein 1
2310042L06Rik	0.0297	0.62	0.56	0.57	0.74	0.43	Hypothetical protein LOC76457
Btd	0.00293	0.63	0.58	0.57	0.74	0.67	Biotinidase

LOC24442	0.0383	0.63	0.55	0.63	0.71	0.65	Similar to hypothetical protein FLJ23749
4833428M15Rik	0.0414	0.64	0.60	0.80	0.52	0.06	Unclassifiable
Pvr	0.0383	0.65	0.67	0.55	0.74	0.16	Poliovirus receptor
Gas2	0.05	0.66	0.69	0.53	0.77	0.60	Growth arrest specific 2
Osgepl1	0.0275	0.66	0.71	0.56	0.71	0.46	O-sialoglycoprotein endopeptidase-like 1
Ptp1a	0.0254	0.67	0.67	0.72	0.63	0.47	Protein tyrosine phosphatase-like (proline instead of catalytic arginine), member a
4632427E13Rik	0.0395	0.67	0.75	0.63	0.64	0.34	Unknown EST
Arid2	0.0213	0.67	0.81	0.67	0.53	0.50	AT rich interactive domain 2
9130401M01Rik	0.0497	0.67	0.61	0.63	0.77	0.61	Hypothetical protein LOC75758
Gtf2e2	0.015	0.68	0.65	0.62	0.78	0.27	General transcription factor II E, polypeptide 2 (β subunit)
2610311I19Rik	0.026	0.68	0.68	0.64	0.72	0.74	Hypothetical protein LOC67180
Zfp68	0.0461	0.69	0.78	0.61	0.69	0.34	Zinc finger protein 68
Cog8	0.0214	0.69	0.65	0.70	0.72	0.34	Component of oligomeric golgi complex 8
Cri1	0.00858	0.69	0.70	0.56	0.81	0.34	CREBBP / EP300 inhibitory protein 1

Gtf2h5	0.0357	0.69	0.88	0.64	0.55	0.70	General transcription factor IIH, polypeptide 5
D830048P04Rik	0.00166	0.70	0.68	0.76	0.66	0.49	Unknown EST
Mtap	0.00197	0.70	0.68	0.72	0.71	0.61	Methylthioadenosine phosphorylase
Cdo1	0.0195	0.70	0.63	0.74	0.74	0.76	Cysteine dioxygenase 1, cytosolic
2410019A14Rik	0.0237	0.71	0.78	0.67	0.67	0.62	Hypothetical protein LOC69746
Smpd1	0.0434	0.72	0.75	0.72	0.70	0.53	Sphingomyelin phosphodiesterase 1, acid lysosomal
1600002K03Rik	0.0365	0.72	0.88	0.69	0.59	0.51	Hypothetical protein LOC69770
Dirc2	0.0391	0.73	0.72	0.75	0.73	0.4	Disrupted in renal carcinoma 2 (human)
Pex12	0.0419	0.73	0.68	0.67	0.84	0.60	Peroxisomal biogenesis factor 12
Tmem33	0.0476	0.73	0.65	0.71	0.83	0.66	Transmembrane protein 33
Dhdh	0.0267	0.73	0.80	0.72	0.66	0.79	Dihydrodiol dehydrogenase (dimeric)
9030624B09Rik	0.00045	0.73	0.68	0.73	0.77	0.61	Hypothetical protein LOC67726
Vhlh	0.043	0.74	0.70	0.84	0.69	0.41	Von Hippel-Lindau syndrome homologue
Agpat5	0.00735	0.74	0.69	0.75	0.79	0.40	Lysophosphatidic acid acyltransferase, epsilon

Txndc1	0.0464	0.74	0.75	0.63	0.85	0.36	Thioredoxin domain containing 1
Armc6	0.0151	0.74	0.74	0.68	0.80	0.60	Armadillo repeat containing 6
Leng1	0.0496	0.75	0.69	0.71	0.86	0.23	Leukocyte receptor cluster (LRC) member 1
Snx5	0.0419	0.75	0.61	0.76	0.87	0.56	Sorting nexin 5
Adck5	0.0179	0.75	0.80	0.64	0.81	0.60	aarF domain containing kinase 5
2610033HO7Rik	0.0404	0.75	0.83	0.74	0.68	0.69	Hypothetical protein LOC75416
Rrbp1	0.0345	0.76	0.66	0.76	0.85	0.37	Ribosome binding protein 1
9030624B09Rik	0.00601	0.76	0.66	0.80	0.83	0.23	Hypothetical protein LOC67726
4631422C13Rik	0.0167	0.76	0.83	0.64	0.82	0.67	Hypothetical protein LOC70799
0610030G03Rik	0.0151	0.76	0.81	0.94	0.53	0.81	Hypothetical protein LOC68385
Scd2	0.0106	0.77	0.74	0.77	0.79	0.22	Stearoyl-coenzyme A desaturase 2
5830453E11Rik	0.0197	0.77	0.71	0.85	0.74	0.40	Hypothetical protein A730008H23
Ikbkap	0.0482	0.77	0.67	0.77	0.86	0.49	Inhibitor of kappa light polypeptide enhancer in B cells, kinase complex associated protein

Nfx1	0.0406	0.77	0.68	0.90	0.72	0.72	Nuclear transcription factor, X-box binding 1
Ncbp2	0.0479	0.77	0.83	0.71	0.76	0.55	Nuclear cap binding protein subunit 2

Relative Expression is the level of gene expression in the presence of dox relative to that in the absence of dox. Genes in red are over-expressed in patients with 11q23 translocations compared to other leukaemias (Armstrong *et al.*, 2002, Ross *et al.*, 2004, Andersson *et al.*, 2005, Kohlmann *et al.*, 2005).

Table A.2 Genes up-regulated by dox in both the conditional and constitutive cell lines.

Gene	P-value	Relative Expression					Product
		Average RE	TRE-ME2	TRE-ME3	TRE-ME6	c-ME1	
Snai1	0.00574	2.07	1.61	2.86	1.75	1.32	Snail 3 homologue (Drosophila)
Rtn3	0.0203	1.99	2.94	1.33	1.69	2.38	Reticulon 3
0610008KO4Rik	7.67E-05	1.91	1.85	2.00	1.89	2.44	Hypothetical protein LOC66039
Wbscr1	0.0292	1.77	2.33	1.67	1.30	1.92	Williams-Beuren syndrome chromosome region 1
Adcy2	0.0407	1.63	1.92	1.82	1.15	3.85	Adenylate cyclase 2
Gpatc2	0.0341	1.52	1.75	1.25	1.56	3.03	G patch domain containing 2
Pscd1	0.0454	1.52	1.67	1.23	1.67	1.67	Pleckstrin homology, Sec7 and coiled-coil domains 1
Sfn	0.0408	1.43	1.56	1.45	1.27	1.33	Stratifin
D330012F22Rik	0.0043	1.42	1.28	1.45	1.52	1.25	Hypothetical phosphopantethiene attachment site containing protein
Dp1	0.0471	1.41	1.25	1.52	1.45	1.56	Deleted in polyposis 1
Cct4	0.0333	1.40	1.33	1.16	1.72	1.32	Chaperonin subunit 4 (delta)

Rab3d	0.0301	1.35	1.32	1.14	1.59	1.18	RAB3D, member RAS oncogene family
2310007D09Rik	0.0343	1.34	1.59	1.09	1.33	1.49	Hypothetical protein LOC71878

Relative Expression is the level of gene expression in the presence of dox relative to that in the absence of dox.

Table A.3 Affymetrix probe ID of genes down-regulated upon loss of MLL-ENL expression.

Gene	Probe ID	Gene	Probe ID
Aldh1l2	1436119_at	Anp32b	1446091_at
Magi1	1433983_at	Amotl1	1455247_at
Hig1	1416481_s_at	Ierepo4	1446512_at
Sdccag33	1427233_at	9530078B04Rik	1433289_at
4921530L18Rik	1441720_at	Taok1	1456975_at
Hoxa9	1455626_at	2510005D08Rik	1435270_x_at
Mtss1	1451496_at	Cdc27	1433848_at
5730405O12Rik	1432843_at	4933432P15Rik	1435694_at
4833442J19Rik	1457915_at	Ap3s2	1436525_at
Senp8	1437307_at	Timm8a	1416346_at
Asf1a	1459882_at	Paip1	1441955_s_at
1700037C18Rik	1430188_at	A730091E23Rik	1446519_at
Elp3	1443452_at	Mettl2	1444716_at
HelB	1419235_s_at	Alg12	1427558_s_at
Pim2	1417216_at	Nomo1	1451170_s_at

Table A.4 Affymetrix probe ID of the genes up-regulated upon loss of MLL-ENL expression.

Gene	Probe ID	Gene	Probe ID
Camp	1419691_at	6230416A05Rik	1453732_at
Pglyrp	1449184_at	Map4k2	1450244_a_at
Ngp	1418722_at	C030046I01Rik	1433932_x_at
Ceacam10	1448573_a_at	Bri3bp	1444439_at
Pram1	1444090_at	Apg7l	1446418_at
1190003K14Rik	1424509_at	Fln29	1428346_at
Ddb2	1425706_a_at	Actn4	1444258_at
Prkd2	1434333_a_at	Aatk	1416936_at
Ceacam1	1422123_s_at	A630020E03Rik	1440196_at
A130086G11Rik	1442805_at	Inpp5f	1445884_at
Hook2	1419350_at	Ypel5	1433593_at
Wdr37	1454823_at	Atrx	1420947_at
Cnn2	1450981_at	Gyg1	1459522_s_at
Cybasc3	1454895_at	D19Wsu12e	1433702_at
Mt1	1451612_at	Slc25a30	1420836_at
Arhgap19	1434911_s_at	Crlf3	1460338_a_at
1190002H09Rik	1446486_at	Sri	1450878_at
Nfatc1	1428479_at		

Table A.5 Affymetrix probe ID of genes down-regulated by dox in both the conditional and constitutive cell lines.

Gene	Probe ID	Gene	Probe ID
3110057O12Rik	1452477_at	Gtf2e2	1448884_at
Selenbp1	1450699_at	2610311I19Rik	1444289_at
2310016C08Rik	1421031_a_at	Zfp68	1417549_at
Egln1	1423785_at	Cog8	1451052_at
C030034I22Rik	1435909_at	Cri1	1448406_at
Gm446	1458718_at	Gtf2h5	1452955_at
A130078K24Rik	1444482_at	D830048P04Rik	1442124_at
Pppr13b	1436590_at	Mtap	1424426_at
Bnip3	1422470_at	Cdo1	1448842_at
Mns1	1419402_at	2410019A14Rik	1454214_a_at
F10	1418993_s_at	Smpd1	1447874_x_at
AI314180	1456255_at	1600002K03Rik	1429091_at
Ang1	1438936_s_at	Dirc2	1454654_at
Ptdsr	1454109_a_at	Pex12	1416259_at
Tiparp	1452160_at	Tmem33	1436028_at
Irak1bp	1431771_a_at	Dhdh	1453487_at
Gpatc4	1449390_at	9030624B09Rik	1424253_at
Sap30	1417719_at	Vhlh	1434708_at
Zfp259	1424465_at	Agpat5	1453257_at
Fndc3a	1443863_at	Txndc1	1437143_a_at
1700022C21Rik	1424999_at	Armc6	1460397_at

2410025L10Rik	1428403_at	Leng1	1425280_at
Frap1	1459625_at	Snx5	1448791_at
2310042L06Rik	1428261_at	Adck5	1436753_at
Btd	1417987_at	2610033HO7Rik	1423991_at
LOC24442	1455665_at	Rrbp1	1452767_at
4833428M15Rik	1431471_at	9030624B09Rik	1451283_at
Pvr	1450295_s_at	4631422C13Rik	1440081_at
Gas2	1450112_a_at	0610030G03Rik	1452132_at
Osgepl1	1435127_a_at	Scd2	1415824_at
Ptp1a	1457434_s_at	5830453E11Rik	1433685_a_at
4632427E13Rik	1429331_at	Ikbkap	1451254_at
Arid2	1454990_at	Nfx1	1428248_at
9130401M01Rik	1452215_at	Ncbp2	1423045_at

Table A.6 Affymetrix probe ID of genes up-regulated by dox in both the conditional and constitutive cell lines.

Gene	Probe ID
Snai1	1456796_at
Rtn3	1443220_at
0610008KO4Rik	1457717_at
Wbscr1	1436637_at
Adcy2	1455462_at
Gpatc2	1457443_at
Pscd1	1418183_a_at
Sfn	1433748_at
D330012F22Rik	1444106_at
Dp1	1442128_at
Cct4	1430034_at
Rab3d	1418891_a_at
2310007D09Rik	1452924_at

05-1691

Research Article

Continuous MLL-ENL Expression Is Necessary to Establish a "Hox Code" and Maintain Immortalization of Hematopoietic Progenitor Cells

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